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PROPERTIES OF CALF THYMUS HISTONE ISOLATED BY THREE DIFFERENT METHODS[1]

L. B. SMILLIE,[2] G. C. BUTLER, AND DAVID B. SMITH

Abstract

Histone of calf thymus deoxyribonucleoprotein was isolated by three different methods: (1) Extraction from nucleoprotein solutions with dilute hydrochloric acid. (2) Extraction from cold saline nucleoprotein solutions with ethanol. (3) Salting out part of the histone, followed by removal of the nucleic acid from the remainder as an insoluble lanthanum salt. Similar yields were obtained in all cases. Measurements of fractional solubility, electrophoretic mobility, and sedimentation rate disclosed the presence of two principal components corresponding roughly to those obtained in the two stages of method (3). Alterations of one component were found to take place in aqueous solutions more alkaline than pH 4.0, resulting in the appearance of artifacts some of which sedimented more rapidly, while others sedimented more slowly, than the original material.

Introduction

Mineral acids have long been used for extraction of histones from deoxyribonucleoprotein (12, 16, 20). Recently, alcohol–salt precipitation of deoxyribonucleic acid (DNA) has been used for separation of histone and DNA (1, 2, 3, 13, 18). Another procedure, the salt method, for isolating histone from thymonucleoprotein (TNP) has now been developed. All three methods give approximately the same over-all yield of protein. The proteins isolated by these methods have been studied by the variable solvent salting-out technique, by sedimentation in the analytical ultracentrifuge, and by electrophoresis. Two main types of calf thymus histone, differing in solubility characteristics, electrophoretic behavior, and sedimentation properties are indicated.

Methods

Analyses were made for nitrogen by the Kjeldahl procedure and for phosphorus by King's (15) method for total phosphorus.

"Purified TNP" was prepared and the percentage extraction of protein nitrogen was calculated as previously described (18). When ammonium sulphate precipitation was used for concentrating the protein, the yield of

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protein nitrogen was estimated by measuring the optical density of the supernatant at 280 mμ.

Hydrochloric acid extraction of histone was carried out at 5° C. as follows: One or two liters of a solution of "purified TNP" in 1 *M* sodium chloride was adjusted to a concentration of approximately 1 mg. of nitrogen per ml. One-half volume of 0.9 *N* hydrochloric acid was added dropwise with constant stirring. The mixture was filtered with suction through Cenco No. 13260 filter paper and after dialysis for 12-hour periods against running tap water and distilled water at 5° C., the filtrate was lyophilized. Histone prepared by such an extraction with hydrochloric acid is referred to subsequently as HCl-histone.

Alcohol–salt extraction of histone from "purified TNP" was carried out at −10° C. as previously described (18). The dialyzed extract was concentrated by pervaporation at 5° C. until it contained 1% of protein. A small amount of insoluble material separated during the concentration and was removed by centrifugation. Histone prepared by alcohol–salt extraction is designated EtOH-histone.

Preparations of HCl-histone or EtOH-histone were separated into two fractions (I and II) by saturation of their 1% solutions with sodium chloride. The precipitate in the case of the hydrochloric acid preparation has been designated HCl-I and in the case of the alcohol–salt preparation as EtOH-I. The proteins contained in the supernatants were similarly designated HCl-II and EtOH-II. After solution, dialysis, and suitable adjustment of concentrations, the solutions were stored at 5° C.

The salt method for histone isolation involved the following procedure: A solution of "purified TNP" containing 1 mg. of nitrogen per ml. was saturated with sodium chloride. The mixture was stirred overnight at 5° C. and then centrifuged at 40,000 × *g* for 2 hours. The relatively clear supernatant was poured off and dialyzed against running tap water overnight. The precipitate was resuspended in saturated sodium chloride by grinding in a Potter–Elvehjem homogenizer. After centrifugation at 10,000 × *g* for 1 hour the supernatant was discarded. The precipitate was again resuspended in saturated sodium chloride and centrifuged at 10,000 × *g* for 30 minutes. The supernatant was discarded and the sediment taken up in a small volume of 0.2 *M* acetate buffer, pH 4.0. Not all of the sediment dissolved. After dialysis against several changes of acetate buffer, the mixture was centrifuged to remove insoluble material. The soluble portion is referred to in subsequent experiments as Salt-I. The supernatant from the first centrifugation, after dialysis overnight, was made 1.5 *M* with sodium chloride. Lanthanum chloride (100 mg. $LaCl_3.7H_2O$ per mg. DNA phosphorus) dissolved in a small volume of a 1.5 *M* sodium chloride solution was added dropwise with constant stirring to the nucleoprotein solution. The gelatinous precipitate of lanthanum nucleate was removed by filtration on a Buchner funnel through Cenco No. 13260 filter paper and the filtrate dialyzed against several changes of 0.2 *M* acetate buffer, pH 4.0, for 24 to 36 hours. Solid ammonium sulphate was added to saturation and after

standing overnight the mixture was centrifuged at 10,000 × *g* for 30 minutes. The sediment, designated Salt-II, was dissolved in a small volume of 0.2 *M* acetate buffer, pH 4.0, and after dialysis against this buffer, a small amount of insoluble material was centrifuged down and discarded. The Salt-I and Salt-II solutions were stored at 5° C.

The "variable solvent" solubility test for protein homogeneity (10, 11, 19) was applied to the histone preparations in the following way. A protein sample was dialyzed against distilled water at 3° to 5° C. for 16 to 24 hours. Solid ammonium sulphate was weighed into 10 ml. volumetric flasks to give final concentrations ranging from zero to saturation. A 3 or 5 ml. sample of the dialyzed protein solution was added, followed by distilled water to the mark. Protein concentrations were such that the unprecipitated controls had optical densities no greater than 1.0. The flasks were shaken vigorously until solution of salt was complete. Volume changes arising from solution of the ammonium sulphate were ignored. The mixtures, after storage at 5° C. for 16 hours, were centrifuged for 15 minutes at 6600 × *g* and the optical densities of the supernatants measured in the Beckmann Spectrophotometer at 280 mμ. Assuming an identical extinction coefficient for all components of the protein, the protein contents of the supernatants were calculated from the optical densities measured at 280 mμ.

A Spinco ultracentrifuge equipped with a Philpot-Svensson schlieren optical system was used for sedimentation experiments. Measurements were made at room temperature in acetate buffer, pH 4.0, and ionic strength 0.2 of which 0.18 was sodium chloride.

Electrophoresis experiments were performed in a Perkin-Elmer electrophoresis apparatus equipped with both the Philpot-Svensson and the Longsworth optical systems for scanning. Most pictures were taken with the latter system. All electrophoresis experiments were done in cacodylate buffer, pH 5.9, ionic strength 0.05, of which 0.03 was sodium chloride.

Results

Recovery of Protein

Typical flow sheets for the three isolation methods showing nitrogen to phosphorus ratios and percentage yields of protein at various stages are to be found in Appendices 1, 2, and 3. Table I shows the over-all yields and the losses incurred during one typical preparation by each of the three methods.

TABLE I

DISTRIBUTION OF PROTEIN DURING ISOLATION BY THREE METHODS

% of total protein nitrogen	HCl method	Alcohol–salt method	Salt method
Not extracted	16.5	2.6	11.9
Lost during dialysis	10.2	16.7	6.2
Rendered insoluble	0	13.0	6.4
Other losses	0	0	7.7
Recovered	73.3	67.7	67.8

The sum of non-extractable histone and histone rendered insoluble is approximately the same for each of the three methods, which may indicate that the protein which is not extracted by one method is identical with the protein rendered insoluble by another. The appreciable loss of nitrogen during dialysis of the alcohol–salt extract may be due to a weakening of the dialysis tubing and an increase in pore size produced by the alcohol. Similar losses have been observed when alcohol solutions of other proteins were dialyzed. In addition, the dialysis sacs became turgid from the entry of water due to the high osmotic pressures of the alcohol and salt solutions used in this method. The final percentage recovery of protein is highest with the hydrochloric acid method probably due to the relative simplicity of the manipulations.

Solubility Studies

Figs. 1 and 2 show the results of the variable solvent solubility tests with the several histone preparations. The rise above 100% initial protein content found at the lower salt concentrations may be ascribed to a visible opalescence which developed in these solutions causing an increase in optical density.

Variable solvent solubility tests were performed on preparations of HCl-histone, EtOH-histone, HCl-I, HCl-II, EtOH-I, EtOH-II, Salt-I, and Salt-II. In Fig. 1, the results for HCl-histone and EtOH-histone were plotted directly while the results for unfractionated histone isolated by the salt method (Salt-histone) were calculated from the curves for Salt-I and Salt-II (see Fig. 2) and the relative yields of these two fractions recorded in Appendix 3.

Evidently, each of the three preparations of histone consisted of two principal components. Furthermore, it can be seen from Fig. 2 that in all three preparations Fraction I corresponded to that portion of the unfractionated histone precipitated between 1.5 and 2.25 *M* ammonium sulphate while Fraction II corresponded to that portion precipitated between 2.25 and 3.5 *M*. It therefore appears that saturated sodium chloride effected a rough separation of the two main histone components.

Nitrogen analyses showed that HCl-I and EtOH-I accounted for 49.1% and 62.1%, respectively, of the preparations of HCl-histone and EtOH-histone. These figures can be compared with 45.7% precipitation by saturated sodium chloride from the nucleoprotein preparation in the salt method. These data together with the solubility curves indicate that the two main components occur in approximately equal amounts in all the thymonucleoprotein preparations studied.

Electrophoretic Studies

The occurrence of two main histone fractions was confirmed by electrophoresis. Patterns of both HCl-histone and EtOH-histone (Fig. 3, A and B) showed the presence of two components. Comparison of these patterns with those obtained with HCl-I, HCl-II, EtOH-I, and EtOH-II (Fig. 3, C, D, E, and F respectively) shows that saturation with sodium chloride effected

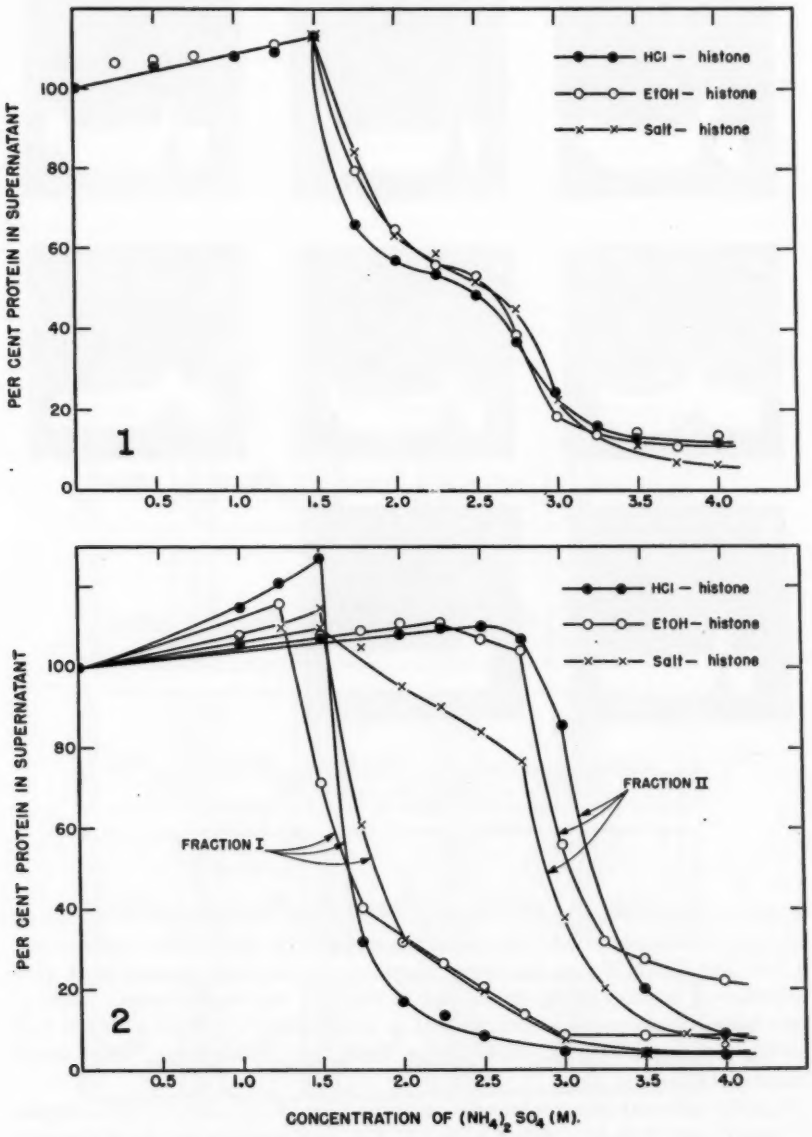

FIG. 1. Solubility curves of histone isolated by three methods.
FIG. 2. Solubility curves of Fractions I and II of histone isolated by three methods.

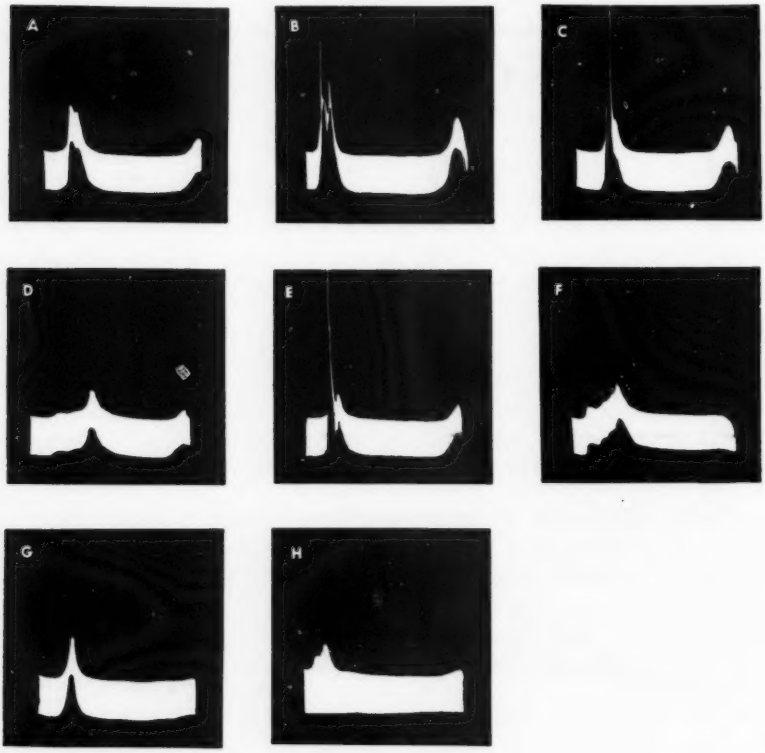

FIG. 3. Electrophoresis patterns of histone preparations: direction of migration from right to left.
(A) Whole HCl-histone.
(B) Whole EtOH-histone.
(C) HCl-I.
(D) HCl-II.
(E) EtOH-I.
(F) EtOH-II.
(G) Salt-I.
(H) Salt-II.

considerable separation. HCl-I and EtOH-I contained the faster migrating component contaminated with a small amount of the slower component. HCl-II and EtOH-II, on the other hand, consisted of the slower peak plus a number of smaller peaks migrating in front of the largest peak. One of these faster components of Fraction II is presumably identical with the fast moving component of Fraction I, but there are, in addition, other small components present.

Slightly different electrophoretic patterns (Fig. 3, G and H) were shown by Salt-I and Salt-II. Salt-I (Fig. 3, G) was apparently homogeneous at pH 5.9 while Salt-II (Fig. 3, H) consisted of two main components, of which the faster peak corresponded to the component of Salt-I. The absence of the small components seen in HCl-II and EtOH-II, in the pattern of Salt-II

may be due to a masking by the larger contamination from the component of Salt-I. This masking has apparently occurred in patterns of unfractionated HCl-histone and EtOH-histone (Fig. 3, A and B). Only after the almost complete removal of the main fast component by saturation with sodium chloride did the spectrum of faster components become apparent (Fig. 3, D and F).

Sedimentation Studies

The histone isolated by the hydrochloric acid method showed a small rapidly sedimenting ("heavy") peak as well as a slow moving ("light") component (Fig. 4, A). The "heavy" component was quite heterogeneous since its boundary spread very rapidly. The "light" component was also not homogeneous and after 155 minutes of centrifugation its boundary had spread quite markedly. HCl-I gave a pattern similar to HCl-histone (Fig. 4, B) except that the "heavy" component made up a larger proportion of the

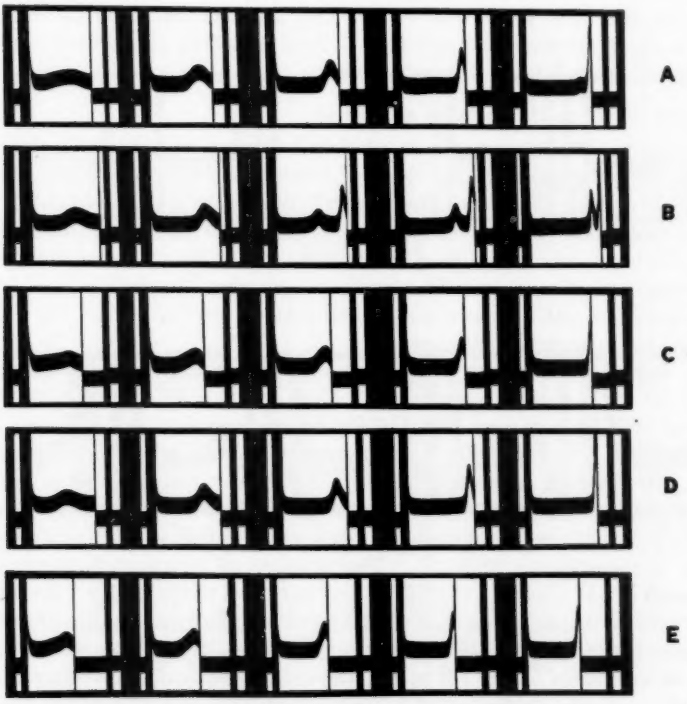

FIG. 4. Ultracentrifuge patterns of histone preparations.
Time intervals after reaching 59,780 r.p.m., from right to left.
(A) HCl-histone, 4, 20, 52, 84, 155 min.
(B) HCl-I, 4, 12, 20, 84, 148 min.
(C) HCl-II, 4, 20, 52, 84, 148 min.
(D) Salt-I, 4, 20, 52, 84, 148 min.
(E) Salt-II, 4, 20, 36, 52, 84 min.

total area of the peaks. The "light" component (sedimentation coefficient 2.1 S) also appeared to be more homogeneous than the "light" component of the unfractionated HCl-histone. HCl-II (Fig. 4, C) showed no "heavy" component and although the peak (sedimentation coefficient 1.2 S) was askew, probably owing to contamination with HCl-I, it was more homogeneous than the unfractionated HCl-histone.

Histone isolated by the alcohol–salt method gave patterns essentially the same as those for the hydrochloric acid preparation, except that the "light" component seemed more heterogeneous. Examination of EtOH-I and EtOH-II showed that the heterogeneous "heavy" component was again confined to Fraction I. Measurement of sedimentation coefficients of the "light" components of EtOH-I and EtOH-II was not attempted because of the rapid spreading of these boundaries.

Examination of Salt-I and Salt-II revealed no "heavy" component (Fig. 4, D and E). In the case of Salt-I the trailing edge of the peak was somewhat askew, whereas with Salt-II the leading edge was askew. This, of course, indicates that the separation of the two main components by saturation with sodium chloride is incomplete. Sedimentation coefficients for Salt-I and Salt-II were 2.3 and 0.9 respectively.

Influence of pH on the Stability of Histone

Although a fresh preparation of Salt-I showed no "heavy" component at pH 4.0, formation of the "heavy" component took place upon storage at pH 7.15 or 8.6. Therefore, the "heavy" component is probably an aggregate of the molecules of the histone of Fraction I. This aggregation was not reversible since returning the pH to a value of 4.0 did not cause the "heavy" component to disappear. The presence of the "heavy" component in preparations of HCl-histone and EtOH-histone indicates that during their isolation some aggregation occurred.

The influence of pH on the formation of this aggregated material was shown by the following experiment. Samples of a 1% solution of unfractionated HCl-histone were dialyzed for 36 hours against the following buffer solutions: (A) hydrochloric acid – veronal buffer, pH 2.45, (B) acetate buffer, pH 4.0, (C) acetate buffer, pH 5.6, (D) cacodylate buffer, pH 6.9, (E) veronal buffer, pH 8.6. After storage at 5° C. for 4.5 days, all of the samples were dialyzed for 4 days against acetate buffer, pH 4.0, and examined in the ultracentrifuge (Fig. 5). The amount of aggregated material was very small at pH 2.5 and 4.0, while it was increased at the higher values of pH.

The sedimentation properties of histones are influenced by the pH of their solutions during storage in another way. When Salt-I was stored in tap water or in buffers of pH 7.0 to 8.5, the component originally characterized by a sedimentation coefficient of 2.3 now sedimented very slowly and formed a very diffuse boundary. This phenomenon was shown by the following experiment. Samples of Salt-I were dissolved in distilled water and dialyzed at 5° C. against the following solutions: (A) tap water for 6 days, (B) 0.3 *N* hydrochloric acid for 18 hours and then acetate buffer, pH 4.0, for 5 days,

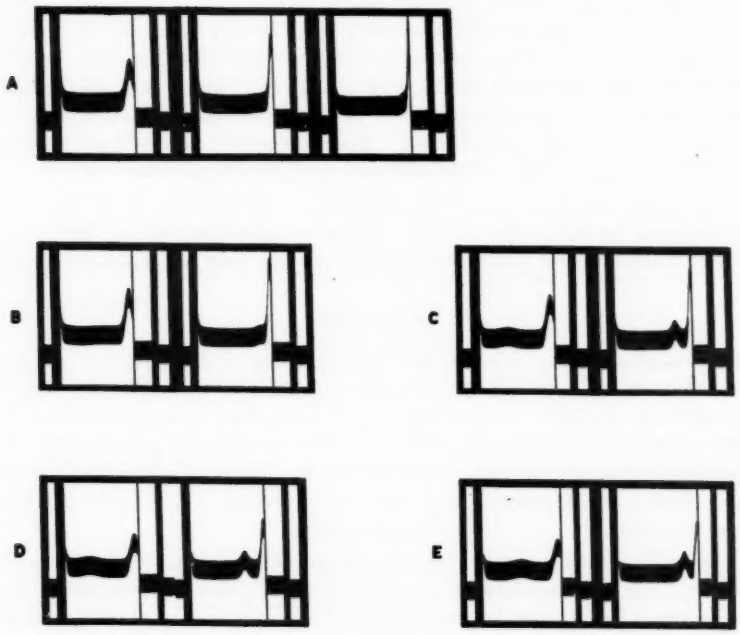

FIG. 5

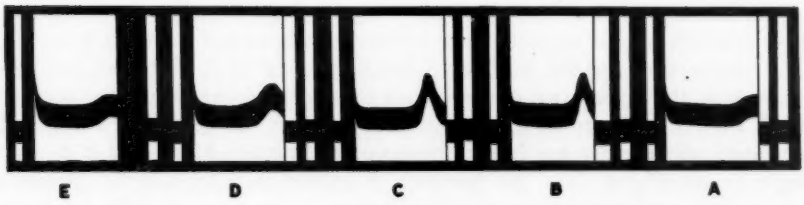

FIG. 6

FIG. 5. Effect of storage pH on the sedimentation of HCl-histone. Time intervals after reaching 59,780 r.p.m., from right to left.
(A) pH 2.5; 1, 4, 20 min.
(B) pH 4.0; 4, 20 min.
(C) pH 5.6; 4, 20 min.
(D) pH 6.9; 4, 20 min.
(E) pH 8.6; 4, 20 min.

FIG. 6. Effect of storage pH on the sedimentation of Salt-I. Time intervals after reaching 59,780 r.p.m.
(A) Tap H_2O, 45 min.
(B) Hydrochloric acid, 45 min.
(C) pH 4.0, 70 min.
(D) pH 7.15, 45 min.
(E) pH 8.65, 45 min.

(C) acetate buffer, pH 4.0, for 6 days, (D) cacodylate buffer, pH 7.15 for 6 days, (E) veronal buffer pH 8.65, for 6 days. Each of the five samples was then dialyzed against acetate buffer, pH 4.0, and examined in the ultracentrifuge (Fig. 6). All pictures were taken 45 minutes after the rotor had reached full speed except in the case of sample (C), which was photographed at 70 minutes. Considerable alteration of the protein had apparently occurred in tap water and at pH 7.15 and 8.65. This alteration of Salt-I had progressed much further than it had for HCl-histone (compare Figs. 5 and 6).

A systematic study of the influence of pH on the stability of EtOH-histone has not been made. The greater apparent heterogeneity of the "light" components mentioned earlier may be due to the long pervaporation period.

Discussion

Many workers have studied the fractionation of histones by a variety of methods (4, 5, 7, 8, 13, 20) and have demonstrated their physical and chemical heterogeneity. In the work just described we have shown them to be readily separable into two main fractions by salting out procedures. It is evident from the solubility and electrophoretic properties of the two fractions that neither is homogeneous and each may consist of a family of closely related proteins.

Amino acid analyses made by several workers (7, 13, 20) indicate that the less soluble histones have a relatively high arginine content and a lower lysine content, while the reverse is found with the more soluble histones. Some preliminary amino acid analyses performed in this laboratory are in accord with this general picture.

Crampton, Moore, and Stein (4, 5) have reported the chromatographic fractionation of calf thymus histone isolated by the alcohol–salt method. Two main fractions, A and B, were obtained which, because of their amino acid content, appear to correspond approximately to our fractions I and II. The recovery of histone was not quantitative and it is probable that this arises from the aggregation of the histone previously reported by other workers (6, 9, 14, 21). This tendency to aggregate was very marked in our Fraction I and only occasionally has it been observed in Fraction II. Luck *et al.* (17) have demonstrated that the presence of guanidine hydrochloride prevents aggregation and permits quantitative elution of the histones from a column of ion exchange resin.

In this work, the isolation of histone by a method involving only treatment with salts has resulted in a preparation in which there is no aggregated material, demonstrating conclusively that the high molecular weight material observed in most histone preparations is an artifact. Histone isolated by this method would seem to be in a less denatured condition than histone isolated by alcohol–salt or hydrochloric acid extraction. It would also appear to be a suitable material for a study of the factors affecting, and the nature of, the aggregative and degradative changes which occur in histone preparations.

The nature of the degradation occurring at pH values above 5.0 is not known. Butler, Davison, James, and Shooter (1) have compared alcohol–salt extracted histone with histone isolated by the acid method and found the former to be more complex electrophoretically. This complexity was less after working at pH 9.0 or with thiol inhibitors. They therefore attributed the complexity to degradation of the histone by cathepsins whose optimal activities are at pH values of 4 to 5. This is in contrast to our findings, which show a greater stability of histone at pH values below 5.0. It is doubtful therefore that these changes in properties at neutral and alkaline pH are due to the activity of an enzyme hitherto described. Luck *et al.* (17) have shown that a similar degradation occurs in the presence of guanidine hydrochloride. Further studies to elucidate the nature of these alterations are contemplated.

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Professor A. F. Graham generously placed an analytical ultracentrifuge at our disposal and Professor C. S. Hanes kindly loaned us the electrophoresis apparatus.

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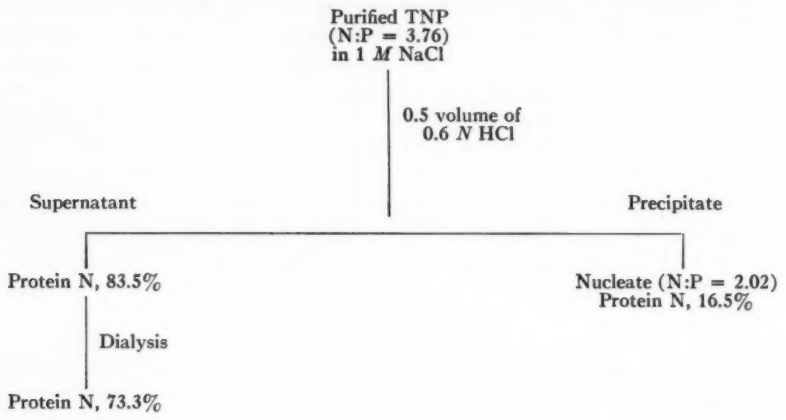
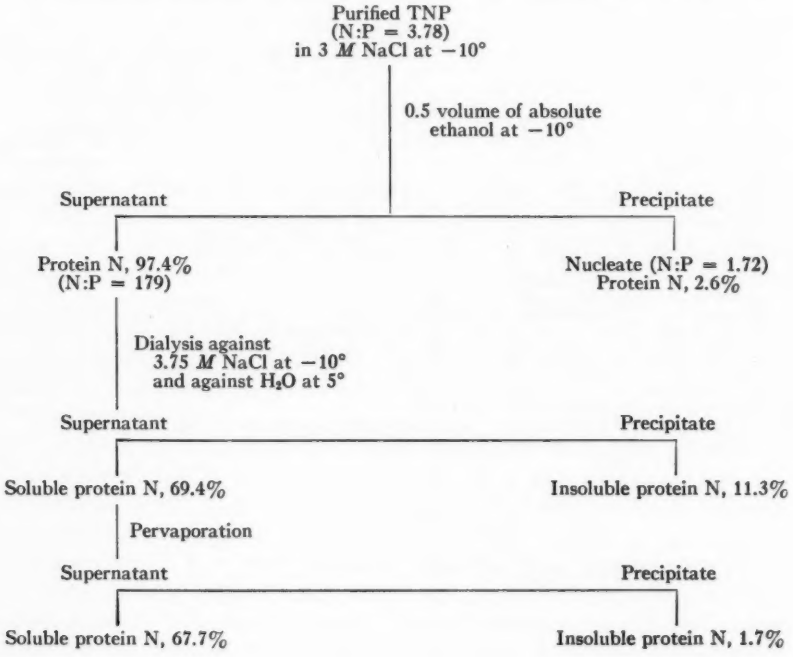
APPENDIX 1

SCHEMATIC DESCRIPTION OF HISTONE ISOLATION BY HYDROCHLORIC ACID METHOD



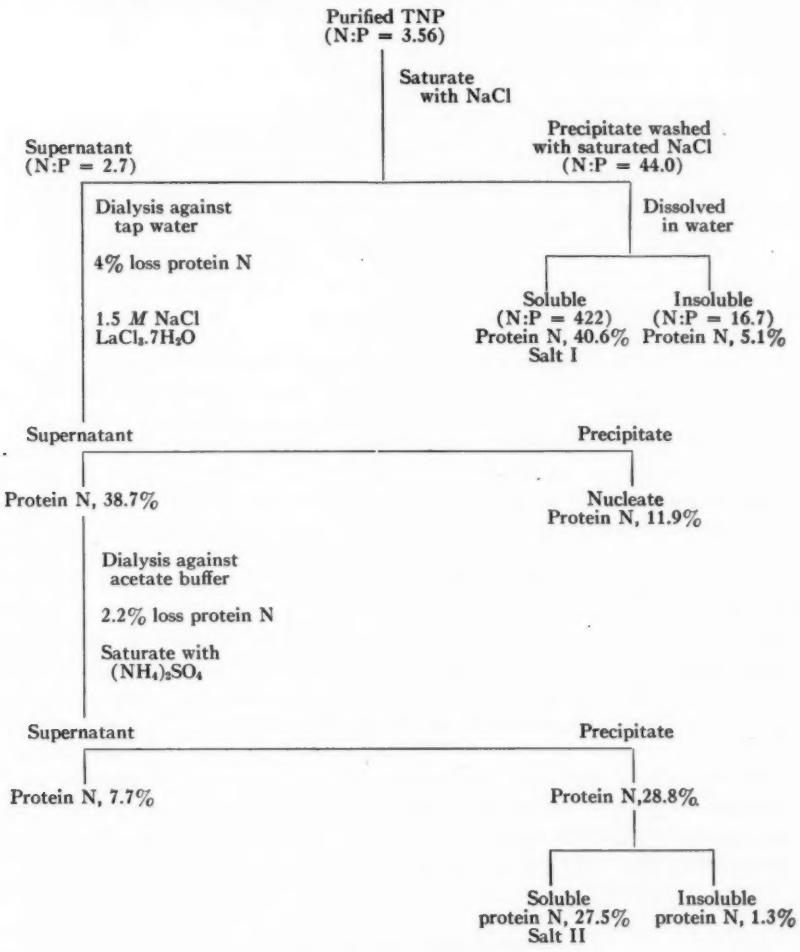
APPENDIX 2

SCHEMATIC DESCRIPTION OF HISTONE ISOLATION BY ALCOHOL–SALT METHOD



APPENDIX 3

SCHEMATIC DESCRIPTION OF HISTONE ISOLATION BY SALT METHOD



STUDIES ON THE ENZYMATIC HYDROLYSIS OF SARIN AND TABUN[1]

PETER A. ADIE

Abstract

The enzymes which hydrolyze isopropyl methylphosphonofluoridate (sarin) and ethyl N,N-dimethylphosphoramidocyanidate (tabun) have been studied. Michaelis-Menten constants and activation energies have been estimated and enzyme stability has been studied. The distribution of the enzymes in mammalian tissues has been examined. It is concluded that from each source the same enzyme is responsible for the hydrolysis of sarin and tabun but that this enzyme shows variations from source to source. That there are mixtures of very similar enzymes, having a composition in amounts characteristic of the source, is also considered possible.

Introduction

The enzymatic hydrolysis of organophosphorus anticholinesterase in general has evoked much interest in the past decade. Two very potent compounds of this type are isopropyl methylphosphonofluoridate (sarin) and ethyl N,N-dimethylphosphoramidocyanidate (tabun). Although Augustinsson and Heimbürger (7) examined the specificity of tabunase ("phosphorylphosphatase"), little mention was made of the hydrolysis of sarin. Their experiments disclosed, however, that tabun and diisopropylphosphorofluoridate (DFP) were probably hydrolyzed by the same enzyme. If this is so then tabun is hydrolyzed by the series of enzymes ("dialklyfluorophosphatases") studied by Mounter *et al.* (10–18). Some of these enzymes may be of the type named A-esterases by Aldridge (4). Mounter states that the rabbit serum dialkylfluorophosphatase is an A-esterase, but according to Augustinsson the enzyme in rabbit plasma which hydrolyzes DFP is probably not (7). In view of additional evidence presented by Mounter, his interpretation of the results obtained is more likely correct.

The kinetics of the enzymatic hydrolysis of sarin and tabun has been examined previously in our laboratory from the point of view of reaction order and stereospecificity (2, 8). The present paper reports further studies of the reaction kinetics of the hydrolysis reactions, together with experiments designed to determine enzyme specificity.

Materials and Methods

Freshly distilled samples of sarin and tabun were used. The sources of enzyme were varied and are noted in the appropriate section of the text. The hydrolysis of sarin and tabun was followed manometrically in a Warburg apparatus. The side arm of the flasks contained the sarin or tabun and the

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main compartment contained the enzyme preparation and 0.025 *M* sodium bicarbonate solution (equilibrated with 95% N_2, 5% CO_2) to make a total volume of 5 ml. The bath temperature and the concentrations of substrate and enzyme varied depending on the type of experiment performed and are noted in the text. Non-enzymatic hydrolysis was followed by replacing the enzyme solution with 0.85% saline solution.

Experimental

1. Determination of Michaelis–Menten Constant

The hydrolysis of sarin and tabun by rat serum at six different substrate concentrations was followed manometrically. The Warburg flasks contained 0.5 ml. diluted rat serum, 0.5 ml. substrate solution (in the side arm), and 4.0 ml. of 0.025 *M* sodium bicarbonate solution. The range of substrate concentrations used in these experiments was limited by the activity of the enzyme and by the non-enzymatic hydrolysis rate of the substrate, which accounted for a considerable gas evolution when molarities above 0.04 *M* were used. The data obtained for sarin and tabun were plotted (Fig. 1) to obtain the Michaelis–Menten constants (Lineweaver and Burke (9)). Each point used in the plot was the b_{30}, i.e. the extrapolated 30-minute value obtained from the initial reaction rate (a_{30}) minus the non-enzymatic control reaction rate (Augustinsson (5)). A Michaelis–Menten constant (K_m) of 7.0×10^{-3} *M* was obtained when sarin was used as a substrate and 7.6×10^{-3} *M* when tabun was used.

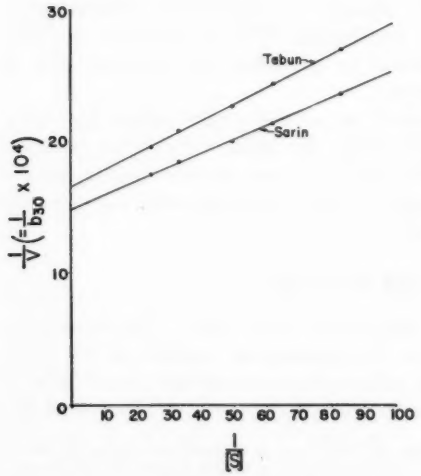

FIG. 1. Plot for the determination of Michaelis–Menten constants.

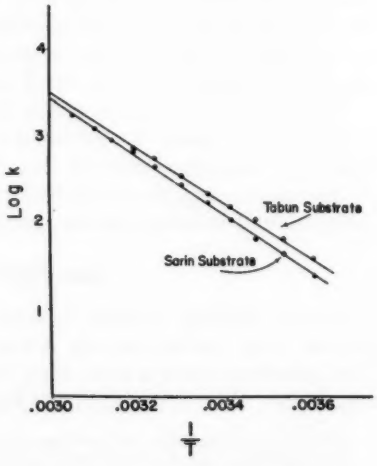

FIG. 2. Plot for the determination of activation energies.

2. Determination of Activation Energies

The rates of hydrolysis of sarin and tabun at different temperatures were investigated using bovine plasma as the enzyme source. A coil containing circulating antifreeze from a cold bath was immersed in the Warburg bath to maintain the lower temperatures. All temperatures were controlled to within $\pm 0.01°$ C. The results of these experiments are shown in graphical form in Fig. 2. Each point is an average of three separate determinations. From the slopes of the curves the activation energies were calculated to be 15,200 cal. when sarin was used as substrate and 14,600 cal. when tabun was used as substrate. At temperatures above 40° C. the experimental results with tabun as substrate were not reliable owing to the high rate of non-enzymatic hydrolysis.

3. Thermal Stability

A marked difference in the rate of heat inactivation of sarinase and tabunase would indicate separate enzymes, but similarity in the rates of heat inactivation would suggest, but not prove, that a single enzyme was acting. Accordingly, bovine plasma was incubated for 14 hours at various temperatures. The plasma was then tested by hydrolytic activity with both sarin and tabun.

The sarinase and tabunase activities of the incubated plasma samples are plotted against the temperature of incubation in Fig. 3. The graph was drawn from the means of two sets of observations from two plasma samples incubated independently at the same temperature. On analysis the differences in activity were not found to be statistically significant.

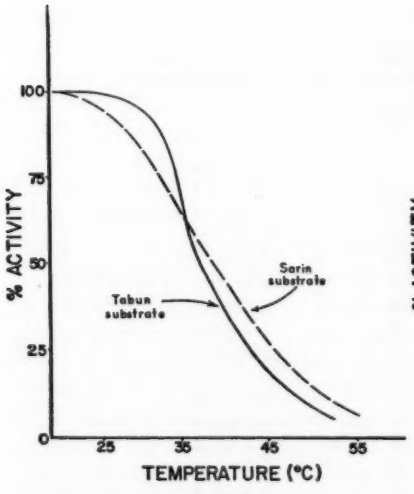

FIG. 3. Graph showing thermal stability of bovine plasma enzyme.

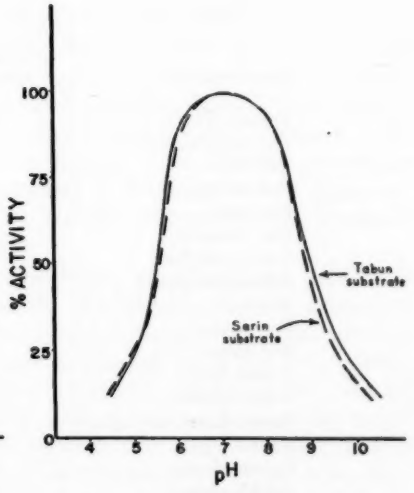

FIG. 4. Graph showing pH stability of bovine plasma enzyme.

4. Stability at Different pH Levels

Because the substrates are unstable above pH 8 and the enzyme denatures around pH 5, it was not possible to obtain reaction rates in a range of pH levels. However, differences in enzyme denaturation after incubation at different hydrogen ion concentrations could indicate the presence of two enzymes. Samples of bovine plasma were incubated for 14 hours at 37.5° C. in Michaelis barbital buffers ranging from pH 4.5 to pH 9.6 (and in a borate buffer at pH 10.5). The samples were dialyzed to remove the buffer and tested for hydrolytic activity with both sarin and tabun. The resulting activities are shown in Fig. 4. The pH graph also was drawn from the means of two sets of observations from two plasma samples incubated independently. In this case very close agreement in activity towards the two substrates was revealed.

5. Purification

In the course of a 1000-fold purification of bovine plasma sarinase hydrolyzing activity increased in a manner similar to the sarin hydrolyzing activity (Adie (1)).

6. Distribution of Activity in Various Mammalian Tissues

Various tissues (homogenized where necessary at 0° C. in a Potter homogenizer) were tested for hydrolyzing activity with both 0.01 *M* sarin and

TABLE I

RATIOS OF INITIAL HYDROLYSIS RATES WITH SARIN AND TABUN SUBSTRATES
(0.01 *M*)

Enzyme source	Number of animals tested	Ratio b_{30} sarin/b_{30} tabun (mean ± standard error)
Monkey liver	5	2.3±0.2
Monkey plasma	5	1.2±0.2
Cat liver	2	4.5±0.5
Cat plasma	2	2.1±0.3
Dog liver	2	4.7±0.4
Dog plasma	2	1.7±0.2
Guinea pig liver	3	2.5±0.5
Guinea pig plasma	3	1.1±0.2
Rat liver	9	4.8±0.2
Rat plasma	9	1.2±0.1
Rat kidney	9	3.5±0.1
Rabbit liver	6	4.3±0.2
Rabbit plasma	6	1.0±0.2
Rabbit intestinal mucosa	2	2.0±0.4
Rabbit brain	2	0.9±0.1
Rabbit kidney	2	1.4±0.3
Rabbit adrenal	2	1.3±0.3
Rabbit spleen	2	1.5±0.3

0.01 M tabun as substrates. From plots of CO_2 production against time, initial reaction rates were obtained and b_{30} values calculated. The ratios of the b_{30} values obtained with the two substrates are shown in Table I.

It can readily be seen from the table that the relative rates of hydrolysis are dependent upon the enzyme source.

Discussion

The Michaelis–Menten constants obtained for rat serum enzyme when using the two substrates are remarkably similar. Similarity of K_m values cannot, however, be taken as evidence of a single enzyme, for such a similarity is not to be expected even when a single enzyme is acting on two different substrates. The constant obtained with tabun and rat serum (7.6×10^{-3} M) is of the same order as that given by Augustinsson, who, using horse plasma and purified enzyme from human blood, obtained values of 3.45×10^{-3} M and 2.15×10^{-3} M respectively (6).

The fact that the energies of activation obtained are similar when the two substrates and one enzyme source are used agrees with the supposition that both substrates are being hydrolyzed by the same enzyme. When the rat serum was incubated under various conditions of temperature and pH and then tested with the two substrates, no statistical difference in stability was observed, indicating the possibility of a single enzyme system for both substrates. The parallel increase in tabunase activity during the purification of bovine sarinase is further evidence of a single enzyme being responsible for the hydrolysis of both substrates by bovine plasma.

The experiments on tissues of various species show that the ratio of sarin to tabun hydrolysis varies both with species and with different organs from the same species. When the b_{30} ratios for all the livers tested are compared with the b_{30} ratios for all the plasmas, it can be seen that in every case the liver ratio is greater than the plasma ratio. Similar variations in dialkylfluorophosphatase from different organs were demonstrated by Mounter (15). Other tests (3) have disclosed that with monkey liver cells the percentage of the total activity within each cell fraction is the same for both sarin and tabun substrates. This suggests that for monkey liver one enzyme is responsible for the hydrolysis of both substrates.

Considering all the evidence so far obtained, one hypothesis possible is that from a single source a single enzyme is responsible for the hydrolysis of both sarin and tabun. There must be, however, a difference in the enzymes obtained from different sources, for each source has its own ratio of tabun hydrolysis to sarin hydrolysis. The possibility of a mixture of very similar enzymes must also be considered. The composition of the mixture would depend on the source. In view of the monkey liver cell fractionation data this possibility is less likely, for there appeared to be no difference in the ratio of sarin to tabun hydrolysis between cell fractions under the experimental conditions used.

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THE INTRACELLULAR LOCALIZATION OF LIVER AND KIDNEY SARINASE[1]

PETER A. ADIE AND JULES TUBA

Abstract

The cells of the liver and kidneys from three species known to be comparatively susceptible to sarin (rabbit, guinea pig, and monkey) and two species known to be less susceptible (rat and mouse) have been fractionated into their cell constituents (nuclei, mitochondria, microsomes, and non-particulate fraction). Each fraction has been tested for sarinase activity. The non-particulate fraction had the highest activity, the microsomes the second highest, the mitochondria the third highest, and the nuclei the lowest. The significance of the differences found in the distribution of sarinase activity in the cell fractions is discussed.

Monkey cell fractions were also tested with ethyl N,N-dimethylphosphoramidocyanidate (tabun) as the substrate. The results were similar to those obtained with sarin, suggesting that the same enzyme was responsible for the hydrolysis of both substrates.

Introduction

It has been shown in experiments in which the individual liver sarinase* levels were compared with individual susceptibility (1) that the amount of sarinase in the liver has an effect on the resistance of individual rabbits to intraperitoneally injected isopropyl methylphosphonofluoridate (sarin). It seems possible that the large differences in the susceptibility of different species to sarin may be due to differences in the amount of sarinase present in the tissues. It is also possible that, if the sarinase were more effective in one fraction than another, the distribution of sarinase within cells would have some bearing on sarinase tolerance. This series of experiments was designed to reveal any variations in the intracellular distribution of sarinase, both between the same organs from different species and between different organs from the same species. It was not designed to reveal differences in total activity between species, as total activity was affected by time of storage of the organs after their removal from the animal. Three species susceptible to sarin on the basis of LD_{50} (rabbit, guinea pig, and monkey) and two less susceptible species (rat and mouse) were chosen for comparison.

Tests on the specificity of sarinase (2) have shown that it is probably an enzyme of the type named "dialkylfluorophosphatase" by Mounter (5) and "phosphorylphosphatase" by Augustinsson (3). Experiments have been included in the present series which show the comparison between the hydrolytic activity of monkey liver cell fractions towards sarin and tabun.

Experimental

1. Cell Fractionation

Animals were stunned by a blow on the back of the head (monkeys were first anesthetized) and killed by decapitation to ensure maximum removal

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* Sarinase is the enzyme which hydrolyzes isopropyl methylphosphonofluoridate.

of blood from the organs. The two kidneys and the liver were removed at once from each animal. The tissues were frozen and stored if they were to be homogenized at a later date. Otherwise the tissues were rinsed in ice-cold isotonic (0.25 *M*) sucrose solution, blotted with filter paper, and cut into smaller pieces with scissors. Approximately 1 g. of the tissue was homogenized with isotonic sucrose in a Potter glass homogenizer, which was cooled in crushed ice, and the homogenate was made up to a volume of 10 ml. with the sucrose solution. One milliliter of the homogenate was put aside for estimation of total enzyme activity of the unfractionated tissue. The remaining 9 ml. was centrifuged at 2000 r.p.m. (6000 *g*) at about 2° C. for 10 minutes. The supernatant liquid was decanted and the first sediment (*nuclear fraction*) was resuspended and washed twice with cold sucrose, and the washings, after centrifugation, were added to the first supernatant. The combined supernatants were then centrifuged at 9500 *g* at 2° C. for 10 minutes. The second sediment (*mitochondrial fraction*) was washed twice. The *microsome fraction* was obtained by centrifuging the remaining supernatant and washings at 100,000 *g* in the Spinco preparative centrifuge at about 8° C. for 1 hour. The microsomal gel was not washed because it was well packed and difficult to resuspend. All three particulate fractions were resuspended and made up to a final volume of 10 ml. with ice-cold sucrose solution. In the case of the microsomes this required the use of the homogenizer. The final *supernatant* liquid, which contained the *non-granular fraction* of the cytoplasm, was made up to a final volume of 25 ml. with sucrose solution.

2. Sarinase Determination

Measurements were obtained in duplicate by a conventional manometric method (6) in which acid formation resulting from sarin hydrolysis was measured in terms of carbon dioxide released from bicarbonate-buffered medium. The dilution of each cell fraction was governed by both the method of fractionation and the activity of the fraction. Each flask contained 0.5 ml. of diluted enzyme preparation.

3. Protein Determinations

Protein nitrogen was determined by the micro-Kjeldahl method and steam distillation.

Results

The specific sarinase activities obtained are shown in Table I. Listed under each specific activity is a figure showing the percentage that this fraction contributes to the total activity.

Table I shows a high degree of variation in individual determinations of the total specific activity. This variation is mainly due to differences between individual animals; some variation was contributed by deterioration during storage. It was not always possible to perform the sarinase determinations at a fixed time after the animal was killed. In general the storage time was 16 hours. The maximum storage time was 64 hours, during which a loss in activity of up to 30% was noted. These variations are reflected in the high

TABLE I

SARINASE ACTIVITIES OF CELLULAR FRACTIONS

Species and organ	Number of animals	Nuclei	Mitochondria	Microsomes	Supernatant
Rat liver	9	469±152*	1100±253	1639±246	2774±256
		2.2±0.6†	12.0±2.2	20.6±2.3	66.0±20.7
Rat kidney	9	123± 70	627± 79	362± 50	1243±111
		2.1±0.7	13.6±2.1	12.9±1.1	69.8±1.6
Mouse liver	6	545±118	569±107	531± 69	1915±138
		5.8±1.3	8.5±1.2	15.6±1.9	70.1±2.1
Mouse kidney	6	40± 14	104± 29	239± 57	572±234
		3.1±1.3	5.6±1.5	15.1±3.7	76.1±4.1
Rabbit liver	4	612± 96	603± 76	1147±134	1554±255
		8.1±1.2	7.1±1.4	25.4±3.7	58.4±5.4
Rabbit kidney	4	374±156	486± 81	606± 79	920±127
		6.2±3.3	9.3±0.9	23.5±2.7	60.0±0.9
Guinea pig liver	6	267± 31	256± 46	506± 42	611± 92
		9.2±0.8	8.7±1.1	28.5±5.1	53.6±4.8
Guinea pig kidney	6	133± 50	252± 68	363± 56	630± 59
		7.7±2.8	13.5±1.9	20.5±2.2	61.5±2.6
Monkey liver	5	62± 28	178± 27	269± 83	971±274
		4.4±2.0	15.9±4.2	14.6±5.1	65.0±8.5
Monkey kidney	5	111± 20	352± 92	294± 36	833± 80
		7.0±1.9	16.8±2.5	17.2±1.6	59.1±2.4

* μl. CO_2 per mg. protein N in 30 minutes ± standard error.
† Per cent the fraction contributes to the total activity ± standard error.

standard errors of the mean specific activities. When the sarinase activity for each fraction is listed as the percentage of the total activity in the sample used, the individual differences in total enzyme level are eliminated. A test made with fresh and stored tissue showed that the relationship between fractions was not affected by storage.

In all cases the soluble fraction (supernatant) had the highest mean specific sarinase activity. The microsome fraction usually had the next highest specific activity. Exceptions were rat kidney, mouse liver, and monkey kidney microsomes. The nuclei in general had the lowest activity. In many animals the activity of kidney nuclei was zero. Because intact cells are sedimented with this fraction (4) the mean activity recorded for the nuclear fraction is probably too high. The activity of the mitochondrial fraction was a little higher than that of the nuclei. In no case was it zero. The percentages that the fractions contribute to the total activity vary considerably from the relative specific activities. This, of course, is due to the variations in the protein content of the fractions. The supernatant fraction is shown to contribute most to the total activity of the cell but this fraction has the highest protein content.

The activities of the monkey liver preparations in the hydrolysis of tabun (ethyl N,N-dimethylphosphoramidocyanidate) were tested in the same

manner as with sarin. To eliminate individual variations in total activity the activities of the fractions were compared on the basis of the arithmetic means of the percentages of total activity contributed by each fraction. The results from five monkeys were used to obtain the means. Table II shows this comparison.

TABLE II

COMPARISON OF SARIN AND TABUN HYDROLYSIS BY MONKEY LIVER

Cell fraction	Per cent the fraction contributes to the total activity ± standard error	
	Sarin substrate	Tabun substrate
Nuclei	4.4±2.0	4.1±1.8
Mitochondria	15.9±4.2	16.2±4.6
Microsomes	14.6±5.1	15.3±2.1
Supernatant	65.0±8.5	64.4±5.4

It can be seen that no significant difference was found in the relative activity of any fraction towards the two substrates.

Discussion

There appear to be species differences in the distribution of sarinase in cell fractions and also differences in distribution in the cells from different organs in the same species. The amount contributed to the total activity by the supernatant fractions of tissues from "resistant" species (Table I) is in every case higher than the amount contributed by the same fractions in "non-resistant" species. This difference, however, is slight and is not statistically significant except between the mouse on the one hand and the rabbit and guinea pig on the other. When specific activities are compared the difference is not significant. Species differences in total activity were not found to be related to species resistance. However, as total activity is affected by storage of the tissues, the experiment was not a suitable one to reveal such differences.

The comparison of the hydrolysis of sarin and tabun shows clearly that the activity towards each substrate is similar for each fraction. This suggests that the same enzyme is responsible for the hydrolysis of both substrates.

Acknowledgments

Thanks are due to Mrs. Marguerite Dickson and Mr. A. C. Merriman of the Physiology Section, Suffield Experimental Station, for their able technical assistance.

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STUDIES ON THE AMINO ACIDS IN THE BLOOD PLASMA OF SCORBUTIC GUINEA PIGS[1]

P. V. RANGNEKER[2] AND L. P. DUGAL

Abstract

A comparative study is made on the plasma amino acids in scorbutic and normal guinea pigs. It is observed that there are some striking differences in the assortment of amino acids obtained. Arginine, threonine, hydroxyproline, and cystine are absent in the plasma of scorbutic guinea pigs while they are found to be present in the plasma of normal animals. A few amino acids have been estimated quantitatively. Plasma values of leucine, valine, alanine, glutamic acid, serine, and glycine are lower and the values of phenylalanine, lysine, and histidine are higher in scorbutic animals as compared with the respective values in normal ones.

Introduction

Mitchell (6) in 1943, in a classical review, discussed the role of ascorbic acid in the metabolism of amino acids. This included a breakdown in the metabolism of phenylalanine and tyrosine in ascorbic acid deficiency and, in addition, several *in vitro* chemical reactions between ascorbic acid and amino acids for which physiological counterparts have not been established. On the other hand, it is well known that ascorbic acid is an essential vitamin for the normal growth in guinea pigs and primates including man.

That the formation of proteins of different types is dependent on the availability of the requisite amino acids which go into their composition is known. Christensen and Lynch (1) have reported that, in ascorbic acid deficiency in guinea pigs, there is a decrease in the glycine and glutamine of skeletal muscle and in the glutamine of liver. Wohlbach and his co-workers (5, 10, 11) have also demonstrated that the formation of collagen is dependent on the presence of ascorbic acid. It was thought therefore that it would be worth while to know if the scorbutic condition has any effect on the concentrations of plasma amino acids. In this paper the plasma levels of a few amino acids in normal and scorbutic guinea pigs are given.

Materials and Methods

Ten male guinea pigs weighing around 400 g. were divided into two groups of five each. One of the groups was fed rabbit chow and water only. The animals in this group gained weight during the first 25 days and this was followed by a distinct loss in weight for the next 22 days. The loss in weight (from the initial weight) observed in these five animals ranged between 142 and 147 g. The five controls had their diets supplemented with 10 mg. of sodium ascorbate given by intraperitoneal injection daily.

[1] Manuscript received July 12, 1957.
Contribution from the Department of Biology, University of Ottawa, Ottawa, Canada. This work was supported by a grant from the National Research Council of Canada.
[2] National Research Council Postdoctorate Fellow.

Technique

Identification of the various amino acids in the blood plasma was done by running two-dimensional chromatograms. The details of the procedure are described in another paper by the present authors (7). Rutter's technique (9) of circular chromatography as modified by Giri and Rao (3) was employed for the estimation of a few amino acids which showed clear separation on the chromatograms. The bands of the respective amino acids on the chromatogram were cut out and eluted in 3.8 cc. of 75% ethanol, and the transmittance for each amino acid was measured on a spectrophotometer at a given wave length after adding 0.2 cc. of 0.1% copper sulphate solution (2).

A separate chromatogram for each amino acid was run by spotting varying amounts of the standard solution. The bands were cut out, eluted, and the transmittance for every eluate was obtained as described earlier at a given wave length. A straight line graph was made which related transmittance at a given wave length to micrograms of amino acid applied to the chromatogram. The figure for actual concentration of a given amino acid in the plasma was then determined from the graph by referring the value of transmittance to the corresponding micrograms of amino acid.

Results and Discussion

The *qualitative* analysis of the plasma of scorbutic and normal guinea pigs showed some striking differences, viz. the total absence of arginine, threonine, hydroxyproline, and cystine in the former. They were all present in the plasma of normal guinea pigs. It is known that threonine is indispensable in promoting growth and maintaining body weight in rat and man (4, 8). In the absence of arginine, growth is possible but only at a subnormal rate (4).

TABLE I

PLASMA AMINO ACID CONCENTRATIONS IN NORMAL AND SCORBUTIC GUINEA PIGS IN 60 μL. OF ALCOHOL EXTRACTS

	Normal guinea pigs receiving 10 mg. of ascorbate daily, average value in μg.	Scorbutic guinea pigs, average value in μg.	Wave length
Leucine	0.55±0.05*	0.41±0.063	520
Phenylalanine	0.19±0.14	0.32±0.14	520
Valine	0.44±0.09	0.39±0.08	530
Alanine	0.29±0.08	0.17±0.07	530
Glutamic acid	1.39±0.05	0.37±0.05	520
Serine	0.47±0.09	0.17±0.01	530
Glycine	1.97±0.55	0.94±0.08	530
Lysine	0.75±0.10	1.10±0.02	520
Histidine	0.58±0.07	0.88±0.02	520
Cystine	0.80±0.30	—	520
Arginine	0.85±0.08	—	520
Total	8.28±1.06	4.74±0.19	

* Standard error.

In scorbutic guinea pigs, it is well known that inanition and inability to retain water in the body seriously affect body weight; moreover, the lack of an indispensable amino acid such as threonine may also be one of the factors which brings about a loss in weight in these animals. Leucine, phenylalanine, tyrosine, valine, proline, alanine, glutamic acid, glycine, serine, histidine, lysine, and asparagine were represented both in normal and scorbutic guinea pigs.

The results of quantitative analysis of a few plasma amino acids of five scorbutic and five normal guinea pigs are tabulated. It is obvious that the plasma levels of leucine, valine, alanine, glutamic acid, serine, and glycine are lower (and significantly so in the last three) in scorbutic guinea pigs than in normal ones. It is quite apparent that in scorbutic animals the decrease in the concentrations of the above amino acids may seriously affect the normal gradients between plasma and tissue cells, which in consequence may impair the normal process of protein synthesis in the body.

The values of phenylalanine, lysine, and histidine in scorbutic guinea pigs are higher than those of normal ones. The lower concentration of plasma phenylalanine in the normal guinea pigs can be very easily explained in the light of our work published elsewhere (7), which suggests that sodium ascorbate supplement aids in the metabolism of phenylalanine and tyrosine.

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ACCURATE MEASUREMENT OF LIQUID INTAKE OF SMALL ANIMALS[1]

R. J. G. Gillespie and C. C. Lucas

Abstract

A device is described for the accurate measurement of the liquid intake of small animals. Examples are given of its usefulness in nutritional studies.

Difficulties are often encountered in the accurate measurement of the volume of liquid consumed by rats or mice in studies of water balance or in investigations involving the intake of other substances (salts, vitamins, drugs, alcohol, etc.) given in the drinking fluid. Complete elimination of spillage seems difficult to attain; evaporation makes accurate measurement of the spilled portion impossible.

The losses from conventional water-bottles, including the Richter type (1) (Fig. 1), although small, may sometimes be considerable. The uncertain performance of these bottles renders their use undesirable when accurate measurement of liquid intake is important. The elongated design of the Richter unit offers problems: the hole in the roof of the cage involves extra work; as the Richter dispenser projects considerably above the cage it limits the number of rows of cages so equipped that may be arranged in one vertical rack.

A more compact unit that (1) minimizes spillage and (2) collects spillage and reduces its evaporation has been designed. The model to be described, which is known as the "Gillespie Drinking Fountain", has been used in about two hundred rat cages for more than three years. Evaporation of liquid from the reservoir is small and relatively constant (about 250 mg./24 hours).

Construction

"Gillespie Drinking Fountains" (Fig. 2) have been made of copper, brass, or stainless steel. They must be rugged enough to stand frequent handling and sterilization and to resist the gnawing habit of rodents. The unit, designed to fit inside any standard cage, consists of a flat rectangular reservoir made from sheet copper (about No. 24 gauge). Figs. 2, 3, 4, and 5 show some of the details of construction. The dimensions are not critical but the capacity of the reservoir should be slightly greater than that of the water bottle adopted for use with it. Near the back of the container a short, wide vertical tube (streamlined tubing O.D. 35 mm. described in commerce as $1\frac{3}{8}$ in., and 35 mm. long) is attached. Into the back of this a piece of streamlined tubing (O.D. 12.6 mm., i.e. $\frac{1}{2}$ in., and about 32 mm. long, see

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Contribution from Banting and Best Department of Medical Research, University of Toronto, Toronto, Canada. This work was supported in part by grants from The Nutrition Foundation, Inc. and The Banting Research Foundation to the Banting and Best Department of Medical Research.

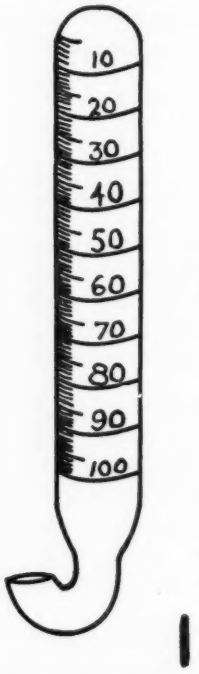

FIG. 1. Diagram of Richter-type watering tube tested in our laboratory: capacity 100 ml., over-all length about 25 cm. (18 cm. projects above the roof of the cage).

Fig. 4) is soldered or brazed at an angle of about 30° to the horizontal. The top of the hole for this side-arm should be 12 mm. below the top of the vertical tube. To carry the hinge for the lid a strip of copper (8 mm. wide and 70 mm. long) is made into a U-shape by right-angle bends 19 mm. from each end; this is attached to the back of the vertical tube at the top, above the side-arm, as shown in Fig. 4. A piece of copper or brass rod 3 mm. ($\frac{1}{8}$ in.) diameter, about 45 mm. long, is soldered to the back of the copper strip. The lid is made from a piece 57 mm. wide and 70 mm. long, trimmed, punched, and bent as shown in the sketches (Fig. 4).

To prevent the animals from pushing the reservoir about in the cage, a short piece of metal rod (3 mm. diameter, not shown in Fig. 4) was soldered in a vertical position to the front face, projecting about 10 mm. below the bottom of the container. This catches in the wire-mesh floor of the cage. To facilitate complete drainage of the reservoir when spillage was being measured, the roof of the reservoir was embossed in the form of shallow diagonal troughs leading from the corners to the center of the vertical tube.

The tab (formed by bending upward the central portion of the back edge of the lid) arrests the opening of the cover, so that the center of gravity of

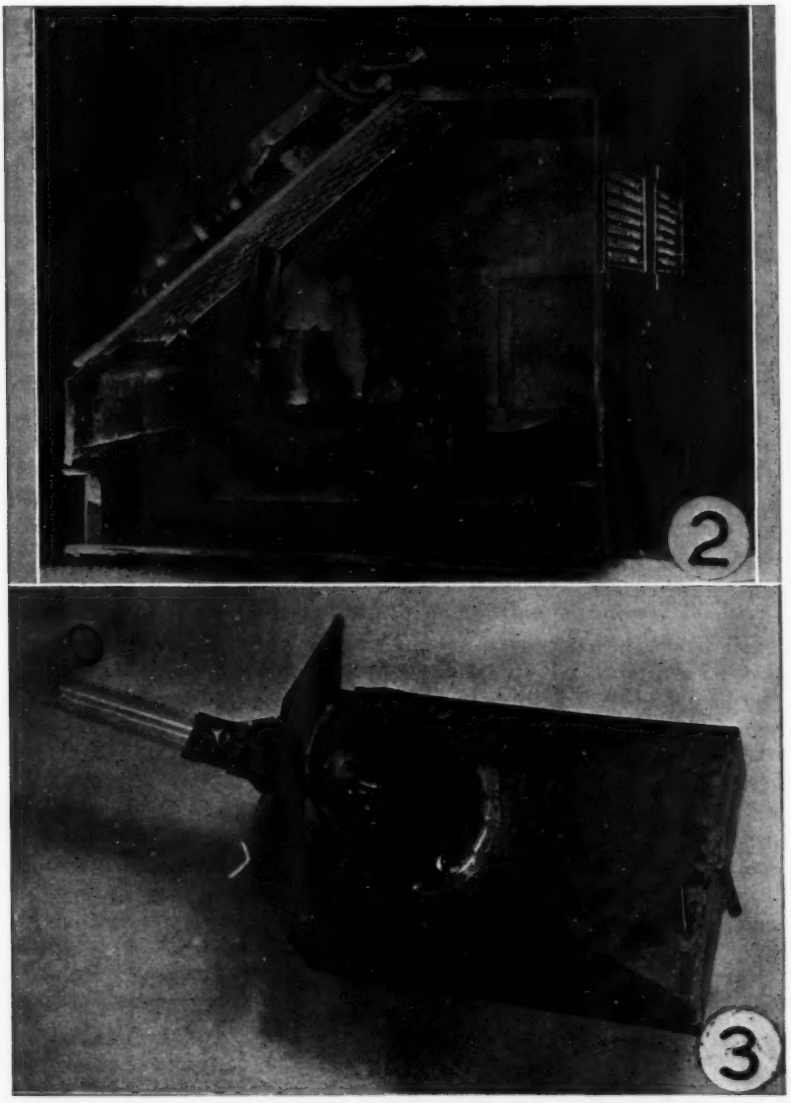

FIG. 2. "Gillespie drinking fountain" mounted in a cage with side cut away to show attachment to water bottle and rat drinking from fountain.
FIG. 3. "Gillespie drinking fountain" with lid held up to show drinking orifice.

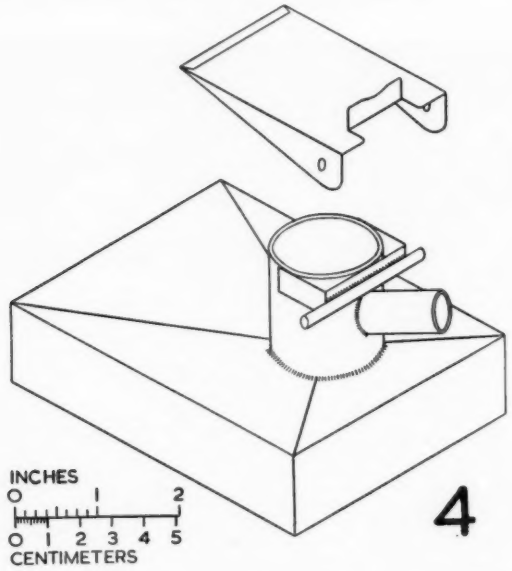

FIG. 4. Orthographic drawing of the "Gillespie drinking fountain" (lid detached to show construction).

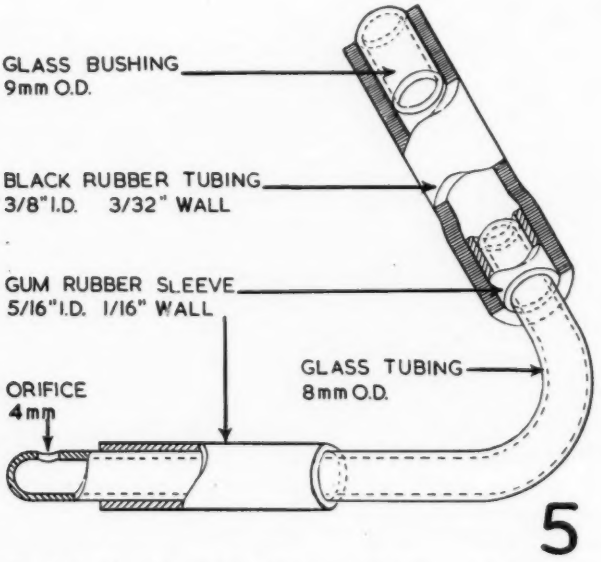

FIG. 5. Sketch showing the details of the connection between the water bottle and the drinking orifice.

the lid will always be in front of the fulcrum; the lid falls down and closes the chamber when the animal is not drinking. The tab, 6 mm. long by 18 mm. wide, bent upwards at slightly less than 90°, must not be cut too deeply or the chamber will not be completely covered. The front edge of the lid, which must project so that the animal can raise it easily with snout or paws, is folded over to give a smooth, rounded edge. Sharp corners are rounded by trimming and filing to prevent injury to the animals. The seating of the lid must be checked carefully to ensure minimum evaporation.

Liquid is conducted from the bottle to the fountain orifice by a piece of glass tubing (Figs. 3 and 5) bent to go through the roof, side, or door of the cage at an angle that will carry it into the reservoir through the side-arm. The internal diameter of the glass and rubber tubing must be large enough to avoid the formation of air pockets that would prevent water from flowing to the orifice. The glass tubing must be at least 8 mm. outside diameter (wall thickness approximately 1 mm.). A sleeve which supports the glass tube in the metal side-arm is made of pure gum rubber tubing; bore 8 mm., so-called 5/16 in. with 1/16 in. wall and length about 32 mm. The orifice at which the water is accessible should be about 4 mm. in diameter. The slope of the glass tube must be such that the bubble of air formed as the rat drinks can travel up the tube to the water bottle, thus permitting more liquid to flow down to the tip. The angle is rather critical: a slope of less than 25° with the horizontal permits air locks to form, but if the angle is too great, the tube will interfere with the mounting and action of the lid.

The bent glass tube is connected with the bottle (in our colony, Queen oval 4-oz. medicine bottles are used) by means of a piece of soft black rubber tubing (I.D. 9 mm., so-called $\frac{3}{8}$ in., with 3/32 in. wall, and about 50 mm. long). Since this does not fit snugly over the glass tubing, a short sleeve about 10 mm. long of amber gum rubber tubing (I.D. 8 mm., so-called 5/16 in. with 1/16 in. wall) is inserted in the end of the black rubber tubing and the combination is slipped over the end of the bent glass tube. The other end of the black rubber tubing is squeezed into the mouth of the bottle. To prevent collapse of this end of the rubber tubing a glass bushing (9 mm. O.D., 1 mm. wall, and about 15 mm. in length) must be inserted. By trial and error, it has been found that to avoid air locks the diameters of glass and rubber tubing given above are rather critical and that the sleeves and bushings are necessary to ensure proper functioning of the units.

Use of the Fountains

Measurement of Spillage

When the water bottle is being disconnected, a straight pull will break the glass tube at the bend. The black rubber tubing connecting the bottle to the glass tip should be rotated back and forth to loosen it from the glass tubing. The contents of the bottle and of the reservoir are poured into separate graduated cylinders (Use funnels!). The sum of the volumes in the two cylinders is subtracted from that offered to get the liquid consumed.

The use of two cylinders is recommended because the liquid in the reservoir is often contaminated by food and dirt; it should be discarded. When only water is in the bottle this, too, could be discarded and a single cylinder would suffice, but when costly substances are being dispensed in the drinking water it is desirable to conserve the clean solution from the water bottle for re-use.

Losses by Evaporation

Three fountains containing 100 ml. of water in the reservoir, left at room temperature (19–23° C.) for 24 hours with lids open, lost 1950, 2000, and 2156 mg., respectively (mean 2035). The importance of the lid is shown by the fact that when it was closed and the side-arm was corked the losses per 24 hours from six fountains were only 195, 197, 247, 258, 301, and 332 mg. (mean 255).

Two fountains containing 100 ml. of dilute (15% v/v) alcohol ($d_4^{20°}$ = 0.97989, corresponding to 11.42% ethanol by weight), left on a bench in the laboratory at room temperature (24–25° C.), lost daily as follows:

No. 1–395, 455, 360, 440, 265, 385, 380 (mean 383).
No. 2–345, 380, 310, 390, 250, 320, 340 (mean 334).

The liquids remaining in the reservoirs ($d_4^{20°}$ = 0.98051 and 0.98031) consisted of 10.97% and 11.12%, respectively, of ethanol by weight. *Alcohol* lost from the fountains was 742 and 549 mg., respectively, corresponding to 27.7% and 25.2%, respectively, of the total weight lost. Over somewhat longer periods, the material evaporating consisted on the average of about 30% alcohol and 70% water by weight.

In prolonged tests, covering an 80 day period, total losses in weight from six reservoirs containing water were 15.46, 15.74, 19.63, 20.45, 23.88, and 26.40 g., respectively (average daily loss 255 mg. or about 10 mg./hour). With dilute ethanol in six reservoirs the losses were 24.22, 24.96, 25.71, 27.06, 32.28, 43.93 g. (average daily loss 374 mg. or about 15 mg./hour). The compositions of the residual liquids varied from 3.8 to 4.7% ethanol by weight.

The average loss (about 200 to 400 mg. per day) may seem large but it should be compared with the complete evaporation of as much as 25 to 50 ml. from the bottom of a cage in 24 hours. Very considerable losses by spillage might go undetected when either the conventional or Richter type water bottles are used. This uncertainty is eliminated when the Gillespie fountain is used. The negligible loss by evaporation when spillage does occur can be corrected for with considerable accuracy.

Training Animals to Use the Drinking Fountain

About six animals are put into a group cage with food; the usual water-bottle tip is replaced by a fountain with the lid wired open. Alert animals quickly discover that drinking water is available from the orifice in the fountain. The unit is left with them overnight for the slower animals to learn

from the smarter ones where to get their drinking water. The device is removed the next afternoon. The following morning the fountain is replaced with the lid closed. Interest in water can be stimulated promptly by offering several rats a drink from the tip of a conventional water bottle (hand-held) for a few moments only. The thirsty animals start investigating the fountain. The experimenter, at this point, carefully raises the lid of the device by means of a piece of wire put through the mesh door of the cage. It is important to allow the animal to slake its thirst partially before proceeding to the next step, viz. lowering the lid very gently onto its head. If the rat is startled by any sudden movement or noise at this stage, it will be apprehensive about returning to the fountain. Avoid dropping the lid on the animal's head or allowing the cover to fall with a clatter. It may be necessary to lower the lid several times onto the rat's head before it will permit the lid to rest thus while the animal is drinking. When a rat does permit this, it will soon start to push the lid up with its snout or, in some cases, use its paw. After one or two rats have been observed to open the lid a few times, leave them with the fountain in the cage overnight so that the other rats may have a chance to learn from their smarter associates. The following morning any rats that are observed to be drinking from the fountain are removed; these trained animals are kept in group cages in which the water is supplied by one of the fountains. Often, however, no animals happen to be thirsty at the moment the experimenter is observing them and much time may be wasted waiting for the rats to display their accomplishments. Remove the fountain overnight to make the animals thirsty once more. The proficient rats may be picked out readily on the following morning when the fountain is again replaced with the lid down. Animals who come over immediately, open the lid, and drink are removed to the group cages containing the trained animals. Occasionally, a rat has been encountered that is too apprehensive or possibly too stupid to learn to drink from the fountain.

Liquid Consumption Determined with Fountains

The water consumption of a number of individual male rats weighing from 200 to 500 g. and eating the stock laboratory diet was measured daily for some weeks. The daily intake varied from 14 to 36 ml.; most values fell between 19 and 30. When 4% of sodium chloride was added to the diet, the average daily intake of water increased to 41 ml. Two groups of smaller male rats (90 to 150 g.) were given dilute (15% v/v) ethanol ($d_4^{30°} = 0.9793$) in place of water for 4 weeks. Their daily intakes averaged 17.0 ml. and 15.1 ml. (equivalent to 1.90 and 1.69 g., absolute ethanol).

Various water-soluble salts or drugs may, of course, be supplied in the drinking water. These fountains make it possible to determine, with considerable accuracy, the daily intake of compounds so supplied. The use of aqueous emulsions in the fountains has not been investigated but we believe that the scope of the device could be greatly extended by such an application.

Acknowledgments

The authors wish to thank Professor C. H. Best for suggesting the problem and providing facilities for its solution. Our best thanks are due to Mr. C. R. Cowan for facilitating the development of the prototype models. Grateful acknowledgment is also made of valuable technical assistance by Mrs. Felicia Dyrkacz with the liquid-intake studies.

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AN UNEXPECTED FACTOR AFFECTING THE ALCOHOL INTAKE OF RATS[1]

R. J. G. Gillespie and C. C. Lucas

Abstract

Measurements have been made of the amount of dilute alcohol (15% v/v) consumed by adult male rats fed an adequate diet. When both water and alcohol were made available, most rats usually chose water. The relative proportions of the two liquids consumed appeared to be influenced by the positions of the food, water, and alcohol dispensers. Had these observations been less complete, the unwarranted conclusion might have been drawn that some of the rats (possibly for genetic reasons) displayed a marked preference for alcohol.

Introduction

In the course of experiments to find out how the ingestion of non-intoxicating amounts of alcohol affects the food intake of adult rats, some unexpected results of environment on liquid consumption were observed. The liquid intake of these animals has been determined using the drinking fountain described in the previous paper (3). One phase of the study was presumed, in the design of the experiment, to permit the rats free choice between tap water and 15% (v/v) ethanol. When some rats consumed much larger volumes of alcohol on certain days than on others it became clear that unsuspected factors can influence the daily intake of one or other liquid when self-selection studies are being conducted. The impression was gained that these differences were due to the location of the fountains in the cage. The fountains were, therefore, deliberately placed for variable periods (from 1 to 7 days) in four different relative positions, and the alcohol consumption of each rat was followed for about 3 months. An early impression that a number of the rats preferred dilute alcohol to water was found to be incorrect. The ease of access to the fountain seemed to be more important than the nature of the contents.

Experimental

The original object of the experiment was to determine how the consumption of alcohol affects the total daily calorie intake, and what percentage of total calories would be accounted for by the alcohol ingested. One group of five rats was given a purified diet* (by calculation, energy value 4.8 Cal./g.) with water to drink. Another group (five rats) was given the same food with dilute alcohol (15% v/v) as the only available liquid (0.8 Cal./ml.). A third group (25 rats) had a choice, since fountains containing both water

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Contribution from Banting and Best Department of Medical Research, University of Toronto, Toronto, Ontario, Canada.

*The purified diet used in this work had the following percentage composition: alcohol-extracted peanut meal (40 mesh) 20, casein 5, fibrin 1, vitamin powder 1, salts 2, corn oil solution of vitamins A, D, and E 1, corn oil 24, sucrose 45.5, choline chloride 0.5. The salt mixture and vitamin mixes were described by Best, C. H., Lucas, C. C., Patterson, J. M., and Ridout, J. H. Can. J. Med. Sci. **31**, 135 (1953).

TABLE I

EFFECT OF ALCOHOL INTAKE ON FOOD CONSUMPTION

Large male rats (mean weight 500 g.) in individual cages fed purified diet and given water or dilute alcohol (15% v/v) to drink for 63 days. Environmental temperature 21 ± 1° C.

Group No.	Vol. of liquid consumed (ml.), av. per rat per day			Food, g.	Total Calories
	Water	Dilute alcohol	Total		
I*	17.0	–	17.0	15.3	74
II†	–	16.7	16.7	11.7	70
III‡	15.7	3.0	18.7	14.2	71

*Five rats.
†Four rats.
‡There were 25 rats in this group; more detailed information about their behavior is shown in Table II.

and dilute alcohol were placed in each individual cage. The average total calorie intakes per day of the rats of Groups I, II, and III were essentially the same (Table I). In another experiment that lasted for over a year the total calorie intake of other rats fed a different purified diet remained relatively constant whether the animals were offered water or dilute alcohol to drink. In both experiments the rats appeared to adjust their calorie intake by reducing their food consumption when calories were obtained from the drinking fluid.

Table II contains data for three typical rats (the largest, modal, and smallest, respectively) of Group III to show the variability of alcohol consumption from day to day and from rat to rat during a period of 2 weeks. The average total daily intake of calories per unit body weight appears to be relatively constant. Seven of the 25 rats, of which No. 830 is typical, consumed little or no dilute alcohol when they had a choice. On only 1 day during the whole 63 day period did the this rat drink more than 1 ml. The majority of the rats, of which No. 812 is typical, consumed a little alcohol almost every day, but they showed a distinct preference for water. Several seemed to prefer alcohol. Rat No. 824 exhibited erratic drinking behavior and a smaller than average liquid intake.

Some observations made by ourselves and others dealing with the preference of rats for dilute alcohol or water as drinking liquid suggested that factors other than those believed to be the ones under study might be influencing the selection by the animal. Too slavish an adherence to that fetish of many investigators, "average" values and standard deviations, nearly let the most interesting fact escape notice. When Experiment I had been almost completed a careful study of the records of certain individual rats caused grave concern. Several of the 'alcoholic' rats suddenly appeared to have become 'teetotallers' and some of the others began to take considerable alcohol. It was considered at first to be an error in recording. When the change in behavior was observed to occur repeatedly, but in an apparently

TABLE II

RELATIVE CONSTANCY OF TOTAL CALORIE INTAKE/G. BODY WEIGHT/DAY WITH VARIABLE CONSUMPTION OF ALCOHOL

	Rat 830, av. wt. 686 g.				Rat 812, av. wt. 508 g.				Rat 824, av. wt. 399 g.			
Interval, days	Alcohol, ml.	Water, ml.	Food, g.	Total Calories	Alcohol, ml.	Water, ml.	Food, g.	Total Calories	Alcohol, ml.	Water, ml.	Food, g.	Total Calories
2	1	42	48.8	235	1	44	43.4	209	15	16	24.7	131
3	0	64	73.5	352	4	72	68.7	333	27	20	37.2	200
2	1	47	48.5	233	1	55	43.0	207	3	33	33.0	161
1	1	17	24.5	118	3	17	19.5	96	9	0	17.7	92
1	0	22	24.3	117	1	21	20.1	97	3	18	14.7	73
3	0	67	72.2	346	0	72	61.6	296	27	12	42.2	224
2	1	40	44.4	213	1	53	40.0	193	3	30	31.0	131
Av./day	0.3	21.3	24.0	115	0.8	23.9	21.2	102	6.2	9.2	14.3	72.3
Av. Cal./g. body wt./day				0.17				0.20				0.18

erratic manner, another explanation had to be sought. A clue to the mystery was supplied when it was noticed that the positions of the fountains seemed to affect the drinking pattern of certain rats to a remarkable extent.

In Experiment II, therefore, a study was made of the effect of the position of the drinking fountains with respect to the food tray and to a doorway made between two compartments, using one rat per large double cage that was partially divided by a partition across it (as shown in the diagrams at the top of Fig. 1). The dispensers were transposed at intervals of 2 to 3 days from Arrangement A to B and vice versa. Later, using the same 25 animals, the arrangement was changed from C to D and vice versa. The daily consumption of food and of each liquid was determined.

The average consumption of alcohol was slightly greater on each of the 33 days when the dispenser containing alcohol was by itself (Arrangement A) and hence possibly more convenient for the rat. Similarly, the average consumption of water was greater on days when the water fountain was alone. The average daily total liquid intake of the rats remained fairly constant at about 19 ml. with a variation that rarely exceeded ± 3 ml. But these *average* values conceal the observations of most interest. The data for individual rats, at first glance seemingly erratic, were recognized to take on, in many cases, a pattern *characteristic of the individual.*

Table III shows the daily variations in the consumption of food, water, alcohol, and total calories by one rat (No. 817) during a typical 50 day period. This rat consistently drank considerably more alcohol when the fountains were arranged as in Diagram A and more water when as in B. Indeed, on 18 days out of 23 with Arrangement A the consumption of water was almost nil (1 ml. or less). This was not due to any genetic peculiarity or dietary deficiency, however, since on 18 out of 27 days with Arrangement B the same rat consumed practically no alcohol.

Table IV summarizes the variations in alcohol consumption of the 25 individual rats that were followed for 82 days. Five rats (811, 814, 818, 830, and 832) were uninfluenced by the positions of the fountains, approximately the same average amounts of alcohol and water being consumed daily regardless of the arrangement, but with many rats the relative positions of the dispensers did control the 'preference' (Fig. 2). Only those values

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FIG. 1. Effect of the arrangement of the dispenser on the self-selection of drinking fluids.

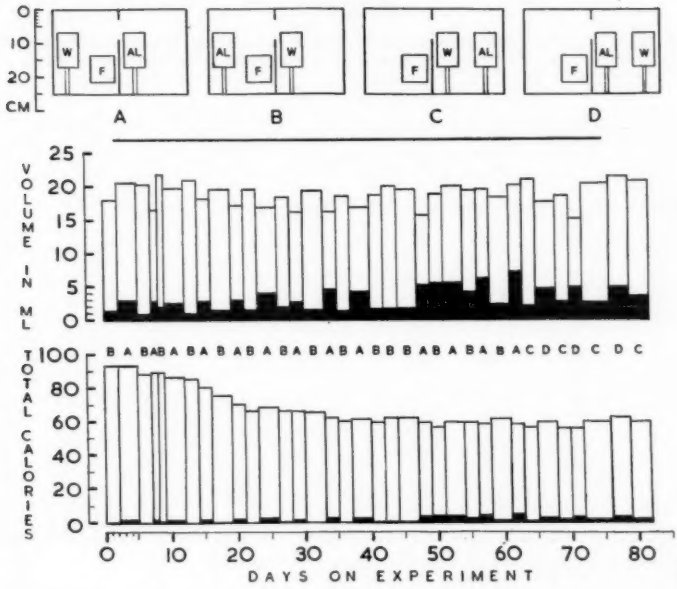
Upper: Scale diagrams showing the four arrangements.

The food dish (F) was always in the front right-hand corner of the left compartment; all the dispensers for water (W) and alcohol (AL) were placed for different periods as shown in Plans A, B, C, and D. Each cage contained one large male rat (average weight 500 g.).

Middle: Average daily consumptions of each liquid, alcohol (black) and water (white), by 25 rats given a choice are shown by the columns.

Lower: Columns show the average daily intake of calories from alcohol (black) and from food (white). Bottom scale shows the duration of the periods.

FIG. 2. Effect of position of drinking fountains in self-selection studies. Figure shows that the choice made by rats between water and dilute alcohol is often strongly influenced by the arrangement of the dispensers. Columns show the daily consumption of dilute alcohol (15% v/v) in black and of tap water in white over a 20 day period on the A–B alternation (see Fig. 1) and for the final 19 day period on the C–D alternation.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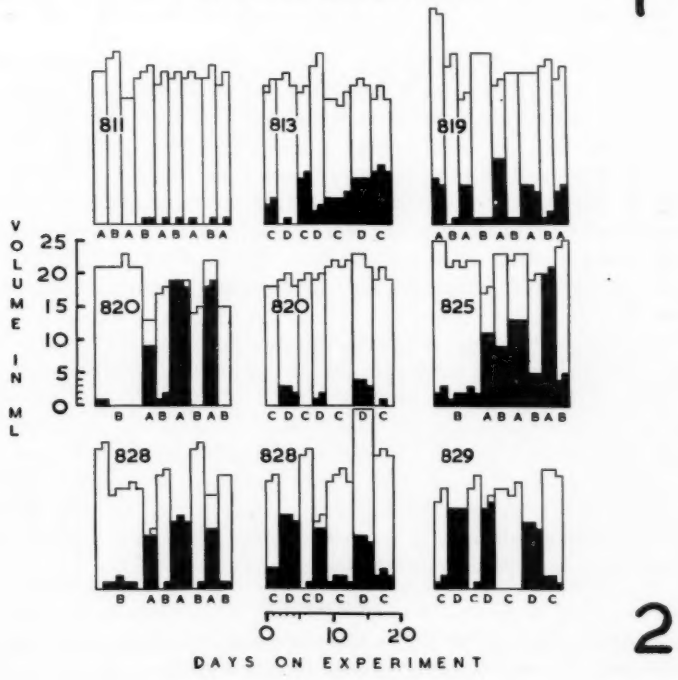
1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DAYS ON EXPERIMENT

2

TABLE III

DAILY INTAKE OF FOOD, WATER, DILUTE ALCOHOL, AND TOTAL CALORIES OF RAT 817 OVER A 50 DAY PERIOD, SHOWING RELATIVE CONSTANCY OF CALORIE INTAKE AND EFFECT OF POSITION OF DRINKING FOUNTAINS ON VOLUME OF LIQUIDS CONSUMED*

Days	Alcohol, ml.	Water, ml.	Food, g.	Total Calories	Days	Alcohol, ml.	Water, ml.	Food, g.	Total Calories
11	*10*	*3*	*9.6*	*54*	36	1	20	14.0	68
12	*10*	*4*	*9.6*	*54*	37	1	20	15.0	73
13	2	18	13.6	67	*38*	*16*	*0*	*13.6*	*78*
14	2	19	13.6	67	*39*	*17*	*1*	*12.8*	*75*
15	*14*	*0*	*8.7*	*53*	*40*	*18*	*1*	*12.8*	*76*
16	*13*	*1*	*8.6*	*52*	41	0	19	13.6	65
17	1	19	11.5	56	42	1	19	14.0	68
18	1	19	13.0	63	43	0	19	13.4	64
19	1	20	13.1	64	44	2	19	13.2	65
20	*14*	*0*	*12.4*	*71*	45	1	19	13.2	64
21	*13*	*1*	*12.4*	*70*	46	1	19	14.4	70
22	2	20	13.5	66	47	1	19	14.4	70
23	2	20	13.0	64	*48*	*16*	*0*	*10.8*	*65*
24	*13*	*0*	*13.0*	*73*	*49*	*17*	*1*	*10.8*	*65*
25	*13*	*0*	*11.0*	*63*	50	1	26	10.8	53
26	*12*	*0*	*12.0*	*67*	51	1	27	10.8	53
27	1	20	14.0	68	*52*	*14*	*5*	*12.0*	*69*
28	1	20	13.7	67	*53*	*15*	*5*	*12.7*	*73*
29	*13*	*1*	*13.8*	*77*	*54*	*14*	*5*	*12.8*	*73*
30	*14*	*0*	*13.7*	*77*	55	4	20	11.4	63
31	1	20	13.8	67	56	4	21	15.0	75
32	1	20	15.2	74	*57*	*19*	*0*	*10.0*	*64*
33	1	20	15.3	75	*58*	*19*	*1*	*11.0*	*69*
34	*19*	*0*	*11.7*	*71*	59	4	15	12.5	63
35	*19*	*1*	*11.8*	*72*	60	5	15	14.9	76

NOTE: Average total daily calorie intake with Arrangement A (23 days), 68.
Average total daily calorie intake with Arrangement B (27 days), 66.
*Figures shown in italics are for days on which Arrangement A (see Fig. 1.) prevailed, i.e. alcohol fountain was alone and presumably more convenient for the rat. Other data are for Arrangement B, with water more convenient.

exceeding 150% of the comparison value in Table IV have been considered to differ significantly, e.g. for Rat 811, 0.36 ml. of alcohol per day on Arrangement B did not exceed 0.30 (Arrangement A) + 50% = 0.45 ml. per day, while for Rat 831, 0.77 ml. per day did exceed 0.30 + 50% = 0.45 ml. per day. On this basis 14 rats regularly drank more alcohol with Arrangement A than B, one drank more with B than A, nine rats drank more alcohol with Arrangement C than D, and nine more with D than C. Preferences tend to become more marked as one descends in Table IV, e.g. Rats 817 and 820 showed an eightfold preference for Arrangement A over B and 30-fold for C over D.

The reason for the apparent preference for alcohol or water may be related to our observation that most rats lift the lid of the fountain with one paw and hold the lid up with it while drinking. If a rat has a tendency to be right- or left-pawed, it might go to whichever fountain was most conveniently located with respect to this inherent preference—unless, of course, this rat had acquired a liking for alcohol. With the possible exception of Rats 825 and 826 (which may be ambidextrous!), none of our animals developed a liking for alcohol sufficient to overcome their reluctance to drink from the fountain when it is inconveniently located (cf. Table IV).

Discussion

The studies of Beadle (1) on biochemical genetics and the reports of Mitchell and Houlahan (9, 10) of partial genetic blocks represent modern extensions of

TABLE IV

TOTAL AND AVERAGE DAILY ALCOHOL CONSUMPTION OF 25 RATS UNDER DIFFERENT ENVIRONMENTAL ARRANGEMENTS FOR 82 DAYS

Drinking fountains placed in cages according to Arrangement A of Fig. 1 for a total of 30 days, B for 33, C for 11, D for 8 days

Rat	Total alcohol, ml.	Mean per day, ml. Arrangement A	Arrangement B	Arrangement C	Arrangement D
		Alcohol intake small (below 120 ml.)			
811	28	0.30	0.36	0.36	0.38
830	36	0.33	0.42	0.45	0.88
832	41	0.57	0.49	0.45	0.38
831	46	0.77	0.30	0.73	0.63
815	46	0.67	0.58	0.55	0.13
818	62	0.77	0.70	0.55	0.88
834	90	1.53	0.82	1.09	0.63
823	103	0.87	0.64	1.09	5.50
835	119	1.37	0.80	1.55	4.00
		Alcohol intake moderate (120 to 299 ml.)			
827	120	1.43	1.57	2.09	0.25
814	184	1.87	1.12	4.00	5.87
812	–	3.20	0.91	*	*
821	203	4.07	1.67	1.09	1.75
813	208	2.13	1.61	5.82	3.38
822	252	5.33	1.91	3.11	0.20
824	277	5.70	1.70	1.09	4.75
		Alcohol intake large (above 300 ml.)			
820	311	8.50	1.00	0.09	2.75
833	316	5.60	2.88	3.91	1.25
819	351	6.68	1.67	6.92	2.38
826	360	3.37	2.81	6.73	11.50
828	381	8.00	1.48	1.82	9.00
816	394	6.40	3.98	5.82	0.88
829	407	2.53	7.15	0.82	11.25
817	597	13.50	1.70	0.64	16.70
825	601	9.67	5.48	9.82	2.75

* Data mislaid.

Garrod's (2) concept of "inborn errors of metabolism". Following up this line of thinking Williams and his colleagues (11–14) in Texas have made the interesting suggestion that alcoholism arises from nutritional deficiences that in turn are genetically controlled. Mardones and co-workers in Chile have independently advanced similar ideas. They postulated an unknown nutritional factor, *N*, which controls the appetite of rats for alcohol (5, 6, 7), and more recently have presented evidence for a hereditary factor in the preference of rats for alcohol (8).

Several years ago, Lester, Greenberg, Smith, and Wu (4) described the effect of including dilute solutions of sucrose and of saccharin as well as of dilute alcohol and water when the rats were allowed to select their drinking fluid. The rats drank the sucrose solutions as well as the dilute alcohol, reducing

the intake of the latter as that of the former was increased. Lester *et al.* concluded that the apparent preference for alcohol in earlier studies was really a response to a demand for calories, not for alcohol per se. They pointed out that their data were not compatible with any narrow concept of alcoholism as due to genetic or nutritional defects.

The data in the present paper support and extend the views of Lester *et al.* The alcohol intake of some rats varied considerably as the position of the fountains was varied, but the total daily intake of calories remained relatively constant. Had we not become aware of the importance to some rats of the location of the fountains and deliberately varied this, we might have inadvertently attributed the differences in alcohol intake to some genetic factor. As it turned out, however, most of the cases of 'alcoholism' observed in our 25 rats given a choice of water or dilute alcohol to drink cannot have had a genetic origin since the 'disease' was 'cured' by changing the positions of the dispensers.

Conclusions

Factors not usually considered may influence the drinking and eating pattern of rats to an astonishing degree.

The relative consumption of two liquids by rats when a choice is offered can be influenced to some extent by the placement of the fountains within the cage. It may be conjectured that some animals are right-pawed and others are left-pawed and that the arrangement of the fountains affects the ease with which the rat may open the lid to get a drink.

Future studies on self-selection of food or drinking-fluid might be more significant if conducted in cylindrical cages. Failing that, scale drawings should be given of the cages used, showing the location of food-dishes and liquid-dispensers so that readers may assess the possible influence of these arrangements.

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DIFFERENCES IN NITROGEN CONTENT IN EXTRACTS OF CHILLED AND NON-CHILLED TISSUES[1]

S. FEDOROFF AND R. ALTSCHUL

Abstract

In an attempt to elucidate the increase of growth promotion of tissue cultures by extracts obtained from tissues chilled before extraction, determinations of nitrogen content were made on (*a*) *fresh* and *chilled* chick embryos, (*b*) extracts prepared from *fresh* and *chilled* chick embryos, (*c*) *fresh* and *chilled* human placenta, (*d*) extracts prepared from *fresh* and *chilled* human placenta.

There was no difference in dry weight, nitrogen content, phosphorus content, and nitrogen : phosphorus ratio between *chilled* and *fresh* chick embryos and *chilled* and *fresh* human placenta. There was, however, a significant difference in the nitrogen content of the extracts prepared from fresh and from chilled tissues. It is suggested that chilling of tissues for 7 days at +4° C. prior to extraction may render the cells more easily destructible during extraction, and thus supply a better yield of nutritive substances.

Introduction

Previously we reported that heart tissue from chick embryos grew more rapidly in cultures containing extracts from embryos which had been chilled for 7 days at +4° C. prior to extraction than in those in which the extract was made from fresh embryos (1). In a corresponding study it was found that strain L cells grew more rapidly in a medium containing a protein-free extract of chilled human placenta than in a medium containing a similar extract of fresh placenta (8).

According to Shipp (14) extracts of frozen chick embryos stored at −12° C. for 6 months are more growth-promoting in tissue cultures than extracts of fresh embryos. Stokes (15), who made extensive studies on the development of *Heracleum sphondylium* L., noticed that embryos of this plant grew more rapidly if the seeds were exposed to low temperatures (+2 to +5° C.) before germination. He suggested that at low temperatures hydrolysis of the endosperm proteins is higher than at room temperature, and thus the rate of accumulation of amino-nitrogen compounds is increased. In addition he found an increased accumulation of certain amino acids which are beneficial for the growth of the embryos.

There are many reports in the early literature claiming that injured tissues produce substances capable of stimulating regenerative processes. These substances have been given various names, e.g. "wound hormones" (5), "necrotines" (10), "cytopoietines" (2), "traumatin" (6), "proliferation-promoting intercellular hormones" (12), "bio-stimulators" (4,9) and most recently, "acroagonines" (3). There is no evidence that all these substances are of the same nature or even related, although it appears that all of them result from sublethal injuries to the cells.

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Contribution from the Department of Anatomy, University of Saskatchewan, Saskatoon, Saskatchewan. This work was supported by a grant from the National Cancer Institute of Canada.

The present investigation was an attempt to ascertain whether or not there are chemical differences between chilled and non-chilled tissues. The main interest centered on possible quantitative changes in nitrogen-containing compounds during chilling of whole embryos.

Methods

Preparation of Chick Embryos

1. Chicken eggs were incubated for 7 days. The embryos were then removed from the eggs and divided into two groups.

2. Lots of four or five embryos of the first group were dried in weighing bottles over concentrated sulphuric acid in a vacuum oven at 56° C. till constant weight was obtained.

3. The embryos of the second group were suspended over sterile water in a museum jar. The jar, sealed with adhesive tape, was kept at +4° C. for 7 days. Aseptic conditions were maintained throughout. The embryos were then removed from the jar and dried in exactly the same way as the embryos of the first group.

4. After they were weighed, the dried embryos of both groups were digested in Kjeldahl flasks with concentrated sulphuric acid. After digestion the material was transferred to volumetric flasks and diluted to volume with distilled water. The nitrogen and phosphorus contents were then determined.

Nitrogen Determination

Water was evaporated from the sample of digested embryos and the digestion was completed with the addition of a mixed catalyst (selenium and copper sulphate – potassium sulphate). After completion of digestion, the procedure of Ma and Zuazaga (13) for micro-Kjeldahl nitrogen determination was followed.

Phosphorus Determination

The water was first evaporated from the sample, and the digestion of the embryos was completed by the addition of hydrogen peroxide. The phosphorus was determined by developing the color with ammonium molybdate and 1-amino-2-naphthol-4-sulphonic acid (ANSA) and the intensity of the color was read in a Coleman spectrophotometer at a wavelength of 660 mμ.

Preparation of Embryo Extracts

Chicken eggs were incubated for 8 days, then divided into three groups. The chick embryos of the first group were removed immediately, and the extract was prepared; those of the second group, retained in the eggs, were kept in a refrigerator at +4° C. for 7 days while the embryos of the third group were removed from the eggs, placed over sterile water in a museum jar, and kept in the sealed jar at +4° C. for 7 days.

The extraction of the embryos was carried out under aseptic conditions in the same manner for all three groups. No bacterial contamination occurred in any of these tests. The embryos were removed from the eggs according to

the technique described by White (16), collected in a Petri dish, rinsed with Hanks' balanced salt solution (11), and homogenized in a hand tissue grinder (A. H. Thomas Co. Specification 4288-B). The homogenate was diluted with Hanks' balanced salt solution in the proportion 1 : 2, and left at room temperature for 2 hours, after which it was centrifuged for 10 minutes at 3000 r.p.m. The supernatant fluid was designated "chick embryo extract".

Preparation of Placenta Tissues and Their Extracts

Freshly delivered placentas were washed free of blood as much as possible with saline, cut into small fragments, and washed again. One portion of the tissue was dried in exactly the same way as described for chick embryos. The second portion of the tissue was put into a museum jar over sterile water and kept at +4° C. for 7 days and then dried in the same way as the first portion.

In another experiment extracts were prepared by the method of Filatov (4,9) from both chilled and unchilled fragments of human placenta.

The nitrogen contents of fragments of placenta and extracts of fragments were determined.

Results

When the nitrogen content of *fresh* and of *chilled* chick embryos was determined and expressed in mg. of nitrogen per 100 mg. of dried tissue, it was found that the difference was very small (Table I). On applying Student's test it was found, however, that the probability of obtaining a difference of such magnitude by chance is less than one in one hundred. In order to explain the slightly greater nitrogen content of the *chilled* chick embryos, the dry weight of the embryos and the total phosphorus were determined. There was no significant difference either in total dry weight (Table I) or in phosphorus content between the *fresh* and the *chilled* chick embryos (Table I). Moreover, the nitrogen : phosphorus ratio was not significantly different between these two groups (Table I). On the other hand, significant differences in nitrogen content between the three types of chick embryo *extracts* were evident, those removed from the eggs before chilling having the highest concentration and those from fresh chick embryos having the lowest (Table II).

TABLE I

ANALYSIS OF TISSUES (MEAN ± S.E.)

Material	No. of samples	Dry weight, mg.	N, % of dry wt.	P, % of dry wt.	N/P
Fresh chick embryos	12	76.0 ± 1.8	10.17 ± 0.05	1.87 ± 0.02	5.43 ± 0.05
Chilled chick embryos	13	71.2 ± 2.2	10.37 ± 0.04	1.90 ± 0.02	5.47 ± 0.04
Fresh placenta	6	406.2 ± 25.3	13.22 ± 0.15	——	——
Chilled placenta	6	399.0 ± 19.6	13.30 ± 0.10	——	——

There was no difference between the nitrogen content of fresh and of chilled human placenta *tissue* (Table I) but *extracts* of chilled human placenta were richer in nitrogen content than extracts of fresh placenta in every case (Table II).

TABLE II

ANALYSIS OF TISSUE EXTRACTS (MEAN ± S.E.)

Extracts prepared from:	No. of samples	N, mg. per 100 ml.
Fresh chick embryos	7	105.6 ± 0.98
Chick embryos chilled inside the eggs	7	121.4 ± 1.90
Embryos chilled outside the eggs	7	136.5 ± 1.23
Fresh human placenta	3	8.63 ± 0.10
Chilled human placenta	3	9.80 ± 0.17

Discussion

The difference in the total nitrogen content of the fresh and the chilled chick embryos is so small that one wonders whether or not it may be a relative increase in the nitrogen content of the chilled tissue rather than an absolute increase. There are at least two possible ways in which a relative increase might have occurred: some volatile non-nitrogenous substances may have formed and been lost during the chilling; or, the fresh tissues may have retained their moisture more tenaciously than the chilled tissue. The total difference in mass of the fresh and of the chilled chick embryos may have gone undetected by the determinations of dry weight and of phosphorus because these determinations have higher standard deviations than the determination of nitrogen.

The significant nitrogen increase in *extracts* prepared from *chilled* embryos as compared to those from *fresh* embryos is probably due to the breakdown of some cells during the refrigeration and also due to the rendering of still intact cells more easily disruptable during extraction. This conclusion is supported by the observation that if the embryos are chilled inside the eggs, the nitrogen increase of the extracts of these embryos is significantly less than when embryos are chilled outside the eggs. When the embryos are refrigerated within the eggs they are bathed in amniotic fluid, which may provide substances capable of retarding the destruction of the cells. Explants of heart tissue from chick embryos maintained at a temperature of +4° C. for 8 days grew well in tissue cultures in 95% of the cases, providing evidence that viability of at least some tissues and cells is not destroyed (7).

Our earlier observation that extracts of chilled tissues are more efficacious in growth promotion of tissue cultures than extracts of fresh tissues might be explained on the basis that chilling renders the cells more fragile, making subsequent extraction of nutrients more nearly complete and/or releases the active substances from inert or non-extractable complexes.

These explanations are supported by the findings of Shipp (14), who worked with frozen chick embryos, and of Stokes (15), who observed increased hydrolysis of endosperm proteins at low temperature.

However, the possibility cannot be eliminated that hypothetical specific substances, for example "bio-stimulators", were responsible for accelerated growth. If one assumes that this increased rate of growth is due to a better nutritional quality of the media rather than to such hypothetical specific substances, it does not exclude the possibility that, during the chilling of tissues, "bio-stimulators" are also produced, as Filatov suggested (4,9). In our own findings of increased growth promotion by extracts from chilled tissues, it remains to be decided whether this effect is related to cellular changes brought about by chilling and manifested by changes in nitrogen content of the extract, or to the action of specific but so far unidentified "bio-stimulators".

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THE EFFECT OF STRESS AND ADRENALECTOMY ON THE RESPONSE TO DICUMAROL IN RATS[1]

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Abstract

The prothrombin time was followed in a large number of rats receiving daily doses of dicumarol. Prothrombin times, using the Schwager–Jaques method, were determined on blood samples obtained from the tail vein. It was found that rats, like rabbits, showed a wide variation in their response to dicumarol at dose levels of 10 and 20 mg./kg. body weight per day but, unlike rabbits, they seemed to develop a tolerance to the drug. This began after a peak prothrombin time was reached and occurred later in rats having a higher prothrombin time, or receiving the higher dose of the drug. Stress, in the form of electroshock or sound-induced seizures, increased the variability of the prothrombin time and significantly increased the mean values with 20 mg./kg. dicumarol. These changes persisted in some rats for at least 48 hours. They were prevented by ether anesthesia. Adrenalectomized rats fed dicumarol showed a marked increase in prothrombin time, did not develop tolerance to the drug after a few days, and died of hemorrhage. An analysis of blood samples obtained by cardiac puncture from adrenalectomized, sham-operated, and normal rats fed dicumarol at a dose level of 10 mg./kg. body weight for 5 days showed that values for the Quick one-stage prothrombin times of the adrenalectomized group were elevated significantly above those of the sham-operated and normal groups. No significant differences, however, were noted in the values for the two-stage tests for prothrombin, hematocrit, or plasma dicumarol levels of these animals.

Introduction

Earlier reports from this laboratory described a marked increase in the prothrombin time of rats following exposure to severe stress procedures of a physical and chemical nature (6) and increased variability of the prothrombin time when these were psychological in character (9). When animals fed dicumarol were subjected to stress, there was a marked increase in mortality and possibly a greater increase in prothrombin time (7). The present experiments were designed to extend the earlier findings. They include groups of animals receiving dicumarol at a dose level of 20 mg./kg. and the use of sound and electroshock while the animals were under ether anesthesia. They also differ from the previous experiments in that blood samples were taken 2 days after stress as well as 1½ hours after stress.

The change in prothrombin time of some rats due to dicumarol was very great. Overman, Field, Baumann, and Link (11) observed little change in the Quick prothrombin time of rats fed dicumarol and for that reason determined prothrombin times with 12.5% plasma, which is a much more sensitive indicator of the action of dicumarol. The most convenient method of obtaining the volumes of rat blood required for determinations of prothrombin time on plasma is by cardiac puncture. However, Jaques, Mogenson, and Fisher

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[3]National Research Council of Canada Student 1956–57.

(7) have found that this procedure seriously interferes with changes in prothrombin time induced by stress. As it was desirable to take blood samples several times to follow the prothrombin time level for a number of days, these were taken from the tail vein of the rat and a modification of the Schwager–Jaques (12) method was used for prothrombin time determinations on whole blood. Campbell, Smith, Roberts, and Link (2) reported that rabbits showed an inherited susceptibility or non-susceptibility to dicumarol, but the same laboratory reported that rats almost always showed some response to dicumarol (11). However, we found in following the prothrombin time for a number of days that many rats developed a tolerance to the drug. Adrenalectomized rats, on the other hand, showed increased susceptibility, and these all died of hemorrhage with dicumarol.

Materials and Methods

Animals

White *rats* of the Wistar strain were used. Source A were rats descended from animals obtained from the Wistar Institute in 1953; Source B were rats obtained from E. G. Steinhilber & Co., Oshkosh, Wisconsin; and Source C animals were obtained from Carworth Farms, Inc., New City, N.Y.

Stress Procedures

Sound-induced seizures were produced by exposing the rats while in their cages to the sound of a Hartmann whistle connected to a source of compressed air (12–15 lb. per sq. in.) and giving a sound of 13,000 cycles per second. The animals were exposed to the sound for from 2 to 4 minutes depending on how quickly seizures occurred.

Electroshock was administered by means of an adjustable alternating current stimulator connected to the 110 volt line. As a means of confinement, the animals were placed in a 12 in. × 12 in. × 5 in. plastic box during the electroshock procedure. Electrode jelly was applied to the rat's ears and small alligator clips were then attached. The circuit was completed by means of a press key.

Adrenalectomy and Sham-operation

The rats were adrenalectomized after the manner described by D'Amour and Blood (3). A sham-operation was performed upon control rats using the technique of the adrenalectomy except that the adrenal glands were not removed.

Blood Samples

Samples of blood were obtained either by *cardiac puncture* or by *venipuncture of a lateral tail vein* while the animals were anesthetized lightly with ether. Two milliliters of blood were drawn from the heart with a 22 gauge needle into a siliconed syringe containing 0.2 ml. of 3.8% sodium citrate and centrifuged to obtain plasma. For tail blood, about 0.1 ml. of blood was drawn with a 0.5 ml. syringe and a 25 gauge needle from a lateral tail vein after the tail had been placed in warm water (45–50° C.) for about one minute to dilate the veins.

Prothrombin Determinations

A modification of the *Schwager–Jaques* method (12) was used. One drop (0.02 ml.) of commercial rabbit brain thromboplastin suspension was placed on a 1½ in. watch glass using a ¼ ml. syringe. To this was added one drop (0.02 ml.) of blood from the tail vein sample. The timer was then started and the mixture stirred with the needle until a clot formed. The timer was immediately stopped and the prothrombin time recorded to the nearest tenth of a second (normal value 26–28 seconds). For more complete studies on the blood, cardiac blood samples were used.

The *Quick one-stage plasma prothrombin time* was determined using a commercial rabbit brain thromboplastin (normal value 13.2–15.2 seconds).

The *two-stage prothrombin determination* was performed using the method of Ware and Seegers (13), and was modified by the addition of barium-carbonate-adsorbed bovine serum to supply Ac-globulin.

Hematocrit readings were made directly from graduated centrifuge tubes. *Plasma dicumarol levels* were determined using a slight modification of the technique of Axelrod, Cooper, and Brodie (1). Toluene was substituted for heptane as solvent in the extraction process.

Dicumarol was supplied by Abbott Laboratories Ltd., Montreal, and was mixed with the standard colony diet (Quaker calf meal pellets). The mixture was prepared in lots of 600 g. with enough dicumarol added to give a dosage of 10 mg. per kg. of body weight (hereafter 10 mg./kg. or 20 mg./kg.). The amount of this prepared food was given according to body weight; a 200 g. rat received 5 g. of food daily. The animals were fed at 9.00 a.m. daily in deep-welled dishes to prevent spilling. Any prepared diet not consumed by the afternoon was weighed, and pellets of the standard colony variety were added so that each rat received a normal quantity of food. This feeding schedule was also followed for the adrenalectomized animals.

The *general plan* of the main experiments was as follows: 161 rats were fed dicumarol (10 mg./kg.) for 8 days and prothrombin times determined on Days 3, 5, and 7. Immediately after this, 85 of the animals that had prothrombin times below 50 seconds were fed dicumarol (20 mg./kg.) for 8 days and blood samples taken for the 3 days as above. One week later, the 85 animals were either adrenalectomized or subjected to sound seizures or electroshock, all again receiving dicumarol (20 mg./kg.). The other 76 animals were either adrenalectomized or used in stress experiments, all again receiving dicumarol (10 mg./kg.). The results in some cases are based on fewer animals than designated above since it was not possible to obtain samples of blood from all rats for each of the 3 days on which tests were made. More extensive studies of blood samples obtained by cardiac puncture were made on 30 rats fed dicumarol (10 mg./kg.), of which 10 were adrenalectomized, 10 sham-operated, and 10 served as controls. In addition, blood samples were obtained from a similar group of 30 rats that had received no anticoagulant.

Results

Prothrombin Time Response of Rats to Dicumarol

The frequency histogram in Fig. 1(*a*) shows the distribution of prothrombin time values of rats on Day 5 when fed dicumarol (10 mg./kg.) for 8 days. The class interval selected is based on the logarithms of the prothrombin times instead of the prothrombin time values themselves. The latter gave a highly skewed distribution and Jaques (5) has pointed out the usefulness of taking log values when reporting clotting times such as the prothrombin time. It is clear from this figure that there was a wide variation of response. The largest number (27%) of the rats showed prothrombin times one class interval above the normal, 54% were two or more class intervals above, 16% gave normal values, and 2%, values shorter than the normal range. A wide distribution of values for the prothrombin time were also found on the other days (Days 3 and 7) when blood samples were analyzed. However, in spite of the variability, two or three patterns predominated. These patterns are shown in Fig. 2(*a*) by the responses of three rats together with the number

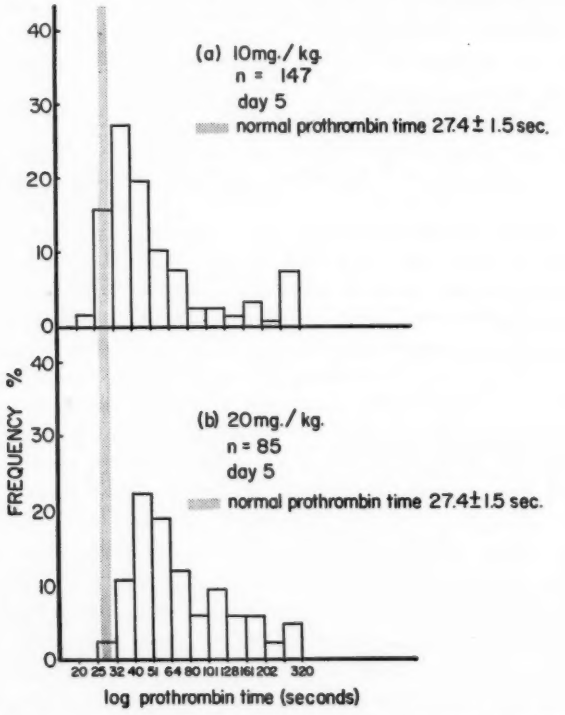

FIG. 1. Frequency distribution of prothrombin times in rats following dicumarol. Dosage, 10 and 20 mg./kg. per day for 8 days. Animals in (*b*) were those with prothrombin times less than 50 seconds in (*a*). Prothrombin times shown for 5th day.
Mean (*a*) 1.7338 ± 0.3021 log seconds. (*b*) 1.8484 ± 0.2614 log seconds.

of animals that showed these typical response patterns. Although some animals had a higher prothrombin time on Day 5, most animals showed a decline after Day 3 in spite of the continued feeding of dicumarol. For the animals fed dicumarol (10 mg./kg.) the prothrombin times corresponding to the mean log values for the third, fifth, and seventh days respectively were 85, 74, and 53 seconds. Using the method for large uncorrelated samples the difference between the means on Day 3 and Day 7 were highly significant ($t = 5.17$, $p < .001$). Although the prothrombin times were not followed in these rats beyond Day 7, results from another experiment, performed at the same time on rats of the same source, suggested that the decline continued until the prothrombin time was nearly back to normal (26–27) seconds. In a group of 22 rats fed dicumarol (10 mg./kg.) for 15 days the prothrombin time on Day 15 was 32.0 seconds (1.4991 ± 0.0732 log seconds). Thus, many of the rats fed dicumarol (10 mg./kg.) in these experiments seemed to develop a tolerance to the drug as manifested by a reduced prothrombin time. This apparent tolerance to dicumarol developed by rats is in contrast to the response of rabbits which do not react in this way (2). An exception to this tolerance to dicumarol and decline in prothrombin time after a peak was reached was found in some animals that showed an increase in prothrombin

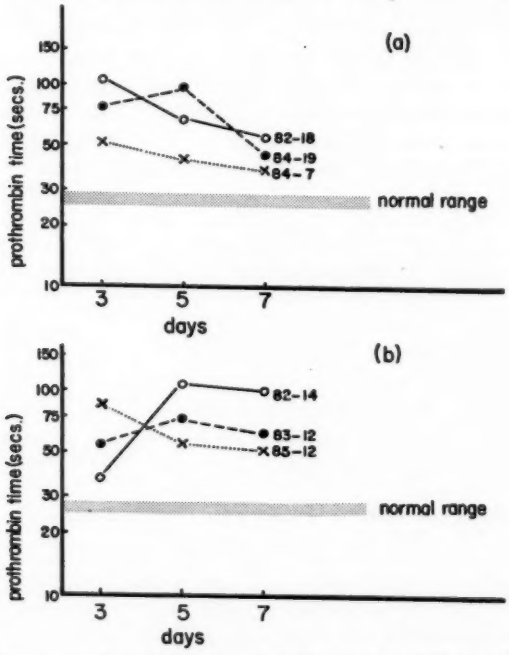

FIG. 2. (*a*) Response of rats fed dicumarol (10 mg./kg. daily). Number of rats whose responses resembled rat 82-18 = 21; 84-19 = 30; 84-7 = 87.
(*b*) Response of rats fed dicumarol (20 mg./kg. daily). Number of rats whose responses resembled rat 82-14 = 15; 83-12 = 46; 85-12 = 24.

time or maintained a prolonged prothrombin time. These rats eventually died of hemorrhage (usually not before Day 5). Of the 161 animals fed dicumarol (10 mg./kg.), 11 died of hemorrhage.

Eighty-five of the animals that had a low prothrombin time by Day 7 when fed dicumarol (10 mg./kg.) were immediately fed dicumarol (20 mg./kg.) for the next 8 days. The frequency distribution of responses for the fifth day of this second period administration of the drug is shown in Fig. 1(*b*). Again, wide variation of response occurred but patterns similar to those with dicumarol (10 mg./kg.) were observed. In Fig. 2(*b*) are shown typical response patterns together with the number of animals whose responses closely resembled these patterns. A larger number of animals had an increase in prothrombin time on Day 5 as compared with response to 10 mg./kg. of dicumarol. However, by the seventh day there was a decline in prothrombin time. Considering the 85 animals together, the prothrombin times corresponding to the mean log values for the third, fifth, and seventh days were 84, 87, and 76 seconds. Since the peak prothrombin time was reached later than when these animals were fed dicumarol (10 mg./kg.) it is necessary to compare Day 7 with Day 5 for some indication that a tolerance to the drug was developed. Using the method for uncorrelated samples, the difference between the means for Days 5 and 7 is significant ($t = 2.43$, $p < .02$).

In the course of these experiments, rats from more than one source were used. The 161 animals so far discussed were all from one source and, as has been shown, a number of these animals had long prothrombin times at both dose levels of dicumarol. Animals from two other sources showed very little change, even with the larger dose of the anticoagulant. Mean prothrombin times for rats from these three sources for Day 5 are shown in Table I.

TABLE I

DIFFERENCES IN SUSCEPTIBILITY TO DICUMAROL (AS GAUGED BY THE PROTHROMBIN TIME) OF WHITE RATS FROM DIFFERENT STRAINS*

Source	*n*	10 mg./kg. dose prothrombin time (log seconds)	*n*	20 mg./kg. dose prothrombin time (log seconds)
A	147	1.7338 ± 0.3021	85	1.8484 ± 0.2614
B	40	1.4896 ± 0.0754	40	1.5706 ± 0.1207
C	15	1.5164 ± 0.0592	15	1.4786 ± 0.0607
Group A compared with group B		$t = 8.17$ $p < .001$		$t = 7.85$ $p < .001$
Group A compared with group C		$t = 7.49$ $p < .001$		$t = 10.87$ $p < .001$

*Values given are mean and standard deviation for Day 5 calculated on prothrombin time in seconds expressed as logs. Samples taken by tail-vein puncture and prothrombin times determined by the Schwager–Jaques method. Prothrombin time for normal control rats was 1.4393 ± 0.0241 log seconds.

The Increase in Prothrombin Time Due to Stress in Rats Fed Dicumarol

The rats used in the stress experiments were the same ones as discussed in the previous section of this paper. After about a week's rest, they were again fed dicumarol (10 mg./kg. or 20 mg./kg.) (depending on the dose previously given) and exposed to stress agents on Day 5. Blood samples were taken 1½ hours and 48 hours later. For calculating the tests of significance the scores (prothrombin times in seconds) were transformed to log values since the distributions were skewed. The mean log values for the groups and the statistical tests are presented in Table II. In lines 1(1) and 2(1) the values for the animals receiving dicumarol are compared with the values obtained for the same animals 14 days and 12 days previously. The values shown were significantly lower statistically than the previous values for animals fed 10 mg/kg. dicumarol but not for those fed 20 mg./kg. dicumarol. The stress of electroshock (lines 1(2) and 2(2)) and sound-induced (line 1(4)) seizures increased the variability of scores 1½ hours after stress. The increase in mean prothrombin time was significant only for the animals

TABLE II

PROTHROMBIN TIME OF RATS FED DICUMAROL AND EXPOSED TO STRESS

Treatment	*n*	Log of prothrombin time*	
		Day 5	Day 7
1. Dicumarol 10 mg./kg.			
(1) Dicumarol alone	11	1.6407 ± 0.0965	1.5410 ± 0.0731
		$F = 10.1$, $p < .01$	$F = 13.7$, $p < .01$
(2) Dicumarol and electroshock	13	1.6952 ± 0.2031	1.5504 ± 0.2055
		$F = 4.43$, $p < .03$	$F = 7.91$, $p < .01$
(3) Dicumarol and electroshock (under ether)	11	1.6691 ± 0.0992	1.5634 ± 0.0499
		$F = 1.10$, $p > .10$	$F = 2.14$, $p > .10$
(4) Dicumarol and sound	6	1.7786 ± 0.2499	1.7370 ± 0.3460
		$F = 6.71$, $p < .02$	$F = 22.41$, $p < .01$
2. Dicumarol 20 mg./kg.			
(1) Dicumarol alone	12	1.9902 ± 0.1412	1.8898 ± 0.1683
		$F = 2.3$, $p > .05$	$F = 1.4$, $p > .05$
(2) Dicumarol and electroshock	15	2.2445 ± 0.2542	2.1583 ± 0.2775
		$t = 3.01$, $p < .01$	$t = 2.63$, $p < .02$
(3) Dicumarol and electroshock (under ether)	13	1.9783 ± 0.1998	1.8873 ± 0.1109
		$F = 2.01$, $p > .10$	$F = 2.30$, $p > .10$

*Values are means and standard deviations expressed in log seconds. Rats were fed dicumarol for 8 days. Blood samples were obtained by tail-vein puncture and prothrombin times by Schwager–Jaques method. Stress was given on Day 5, 1½ hours before taking of blood samples.

Tests of significance compare each group with the control group (dicumarol alone). The control group has been compared with itself for values obtained 2 weeks previously. The student "*t*" test was performed to compare each group but significance using this test was found only in 2(2). For all other comparisons, the ratio of variances (*F* ratio) is reported.

fed the 20 mg./kg. dose and given stress (line 2(2)). Animals receiving dicumarol (10 mg./kg.) displayed a statistically significant increase in variability of prothrombin time after stress (line 2(2)). The prothrombin time of animals given electroshock while under ether anesthesia did not differ from the controls with dicumarol alone (lines 1(3) and 2(3)).

The Increase in Prothrombin Time in Adrenalectomized Rats Fed Dicumarol

All adrenalectomized rats used in these experiments were allowed to rest 1 week before further experimental procedures were carried out. They all received physiological saline ad libitum throughout the experiment. A few adrenalectomized rats were given electroshock and no change was observed in the prothrombin time. This observation was similar to that of previous experiments where it was found that the adrenal gland was necessary for the stress response (change in prothrombin time) to occur (6). However, when adrenalectomized rats were fed dicumarol, there was a marked increase in prothrombin time. The results are presented in Table III. Group 2 (adrenalectomized rats fed 10 mg./kg. dicumarol for 8 days) and Group 4 (adrenalectomized rats fed 20 mg./kg. for 8 days) had prothrombin times significantly longer on Days 3, 5, and 7 than normal controls which received only dicumarol (Groups 1 and 3). All of the adrenalectomized rats fed dicumarol died, whereas, in the controls receiving only dicumarol, there was no mortality. The amount of dicumarol required to provoke these changes in adrenalectomized animals was small. After the second or third day the animals ate

TABLE III

THE EFFECT OF DICUMAROL ON PROTHROMBIN TIME AND MORTALITY IN ADRENALECTOMIZED RATS

Group	n	Treatment	Prothrombin time			Mortality
			Day 3	Day 5	Day 7	
1	6	Dicumarol 10 mg./kg.	1.6834 ± 0.0663	1.6650 ± 0.0993	1.5815 ± 0.0212	0/6
2	6	Adrenalectomy dic. 10 mg./kg.	2.2024 ± 0.1921 t = 5.7 p < .001	2.1514 ± 0.2125 t = 4.3 p < .01	2.1461*	6/6
3	12	Dicumarol 20 mg./kg.	1.9016 ± 0.2130	1.9902 ± 0.1414	1.8893 ± 0.1687	0/12
4	11	Adrenalectomy dic. 20 mg./kg.	2.4048 ± 0.0282 t = 5.2 p < .001	2.3682 ± 0.2128 t = 4.0 p < .001	2.4548*	11/11
5	6	Adrenalectomy	1.4860 ± 0.0240	1.4154 ± 0.0227	1.4255 ± 0.0201	0/6
6	2	Sham-operated dic. 20 mg./kg.	1.6990, 1.6232	1.6721, 1.4771	1.8573, 1.6532	0/2

NOTE: Animals were fed dicumarol for 8 days at the dose specified. Blood samples were taken by tail-vein puncture and prothrombin times determined by the method of Schwager–Jaques. Prothrombin time values given are mean and standard deviation expressed as log seconds. These animals had been fed dicumarol prior to operation.

Tests for significance compare an adrenalectomized group receiving dicumarol with a control group that had received the same amount of dicumarol.

*Blood samples from one animal only. The "t" test was not done on Day 7 since there was only one animal left in each of Groups 2 and 4

practically no dicumarolized food. This is a typical reaction when the prothrombin time becomes very long. In addition, the animals appeared ill. Adrenalectomy alone did not increase the prothrombin time of rats as shown by Group 5, and the increase in prothrombin time was not a result of the surgery since the prothrombin time of the two sham-operated animals (see Table IV also) receiving dicumarol did not exceed that of the normal controls also receiving dicumarol.

An experiment was performed to investigate further the nature of the coagulation changes following the administration of dicumarol to adrenalectomized rats. Blood samples, obtained by cardiac puncture, from 20 adrenalectomized, 20 sham-operated, and 20 normal animals (one-half of each group receiving 10 mg./kg. dicumarol for 5 days) were analyzed using the following determinations; one-stage prothrombin time, two-stage prothrombin level (modified and unmodified), hematocrit, and plasma dicumarol levels. The results are shown in Table IV.

The values obtained for the one-stage test showed no significant difference for the control, sham-operated, and adrenalectomized rats, while the values for the rats receiving dicumarol are in most cases much greater than the values for the corresponding animals which did not receive dicumarol. Now comparing the values for the one-stage prothrombin time for the dicumarol-treated animals, there was no significant difference between the controls and the sham-operated animals but there was, however, a highly significant difference between the values for the one-stage prothrombin time of the control and adrenalectomized animals ($p < .01$), while the difference between sham-operated and adrenalectomized groups was significant at the 0.02 level. The difference between the values for the two-stage prothrombin test was not significant for any treatment except dicumarol. The greatest differences were between the control and adrenalectomized rats receiving dicumarol ($p = .05–.20$) and the sham-operated and adrenalectomized rats receiving dicumarol ($p = .1–.2$). No significant differences were noted in the hematocrit or plasma dicumarol levels of any of the groups.

Discussion

It is evident from the above that there is considerable variation in the increased prothrombin time of rats receiving dicumarol or subjected to other procedures. Some animals show a prolonged prothrombin time, while others show little change. The common use of mean prothrombin times is misleading in these circumstances, leading as it does to very large values for standard deviations. When the values of the log of the prothrombin times are plotted as a frequency histogram a curve is obtained which approaches a normal distribution, reducing the severity of skewness. This justifies the use of means of log values of prothrombin times for comparisons and in fact is essential for a valid comparison of the effect of different treatments on the prothrombin time. The theoretical basis for this is that the prothrombin time, like all clotting times is a rate, the value of which is related to the concentration of reactants logarithmically.

TABLE IV

EFFECT OF DICUMAROL ON THE COAGULATION SYSTEM OF NORMAL, SHAM-OPERATED, AND ADRENALECTOMIZED RATS

Treatment	*n*	One-stage (log seconds)	Hematocrit (% cells)	Two-stage (% normal)		Plasma dicumarol levels (μg./ml.)
				Unmodified	Modified	
Control	10	1.1784 ± 0.0248*	48 ± 2	100 ± 20	100 ± 20	—
with dicumarol	10	1.2792 ± 0.1706	45 ± 2	29 ± 23	45 ± 29	18.6 ± 32
Sham-operated	10	1.2295 ± 0.0200	48 ± 3	89 ± 15	91 ± 14	—
with dicumarol	10	1.4023 ± 0.2880	47 ± 0	24 ± 15	24 ± 21	13.2 ± 14
Adrenalectomized	10	1.1894 ± 0.0003	44 ± 2	89 ± 27	84 ± 27	—
with dicumarol	10	1.9795 ± 0.3480	45 ± 6	10	10	16.0 ± 19

TESTS FOR SIGNIFICANCE (ON ANTICOAGULANT)

Test	Control vs. sham-operated		Control vs. adrenalectomized		Sham-operated vs. adrenalectomized	
	t	*p*	*t*	*p*	*t*	*p*
1-Stage	1.12	0.3–0.2	5.83	<0.01	4.06	<0.01
Hematocrit	0.05	>.8	0.02	>.8	0.08	>.8
Plasma dicumarol levels	0.42	0.6–0.8	0.19	0.8	0.28	0.8
2-Stage prothrombin level (unmodified)	0.3	0.6–0.8	1.57	0.1–0.2	1.43	0.1–0.2
2-Stage prothrombin level (modified)	0.82	0.4–0.6	1.97	0.05–0.1	1.49	0.1–0.2

*Mean values ± 1 S.D.

The experiments reported here demonstrate that the increase in prothrombin times in rats receiving dicumarol depends not only on the dose of dicumarol administered but also on the source of the animals used, previous exposure to the drug, exposure to stress, and presence or absence of the adrenal glands. The variability in the prothrombin time with a standard dose of dicumarol in previously untreated animals can be both of genetic and environmental origin. For example, as judged by other work in this laboratory, the affinity of the liver for the drug and the level of adrenalcortical activity are important. The former not only is markedly different in different species (genetic) but also is affected by the level of vitamin K intake (environmental).

The results of the present experiments with stress in animals fed dicumarol (10 mg./kg.) do not agree entirely with earlier results obtained by us (7) in which a significant increase in prothrombin time occurred in rats receiving dicumarol following electroshock. One reason is that the animals in the present experiment were fed dicumarol for an 8 day period ending 1 week prior to the stress experiment. As a result, they were less affected by the drug during the second feeding period when stress was given. A second reason is that with the whole blood prothrombin time method it is not possible to control temperature and volumes of blood and thromboplastin as accurately as with the modified Quick one-stage method. This would lead to greater variability and would necessitate the use of more animals to demonstrate differences. However, in the present experiment, the increase in the mean prothrombin time was significantly greater at the higher dose level of dicumarol. The mortality data is in agreement with this since 0/13 of the animals given dicumarol (10 mg./kg.) and electroshock died whereas 6/15 given dicumarol (20 mg./kg.) and electroshock died. Since samples of blood were obtained by tail-vein puncture, mortality was much lower than previously where cardiac punctures were used (7).

Table II shows the effect of anesthesia on the prothrombin time following dicumarol and electroshock. It is significant that there is no statistically significant difference in the mean values for rats receiving dicumarol and electroshock under ether and the animals on dicumarol alone. Evidently ether suppresses the increase in prothrombin time due to electroshock. The rats which were given electroshock while under ether anesthesia showed some movement of the upper part of the body while the current was on, but no seizures occurred. The increase in prothrombin time following electroshock may be associated with the activation of certain central structures which have a low threshold of response and which have a close connection with the hypothalamus. The latter in turn may then activate the pituitary.

The effects of stress and of adrenalectomy on the prothrombin time observed in this paper are presumably effects on the coagulation system which are more readily seen when there is already interference with coagulation due to the administration of dicumarol. Both stress and adrenalectomy may affect the liver, the site of production of prothrombin, proconvertin, Ac-globulin, and other factors involved in blood coagulation. In addition, these procedures produce a reduction in capillary resistance (8) which can

contribute to a further increase in prothrombin time through the escape of plasma proteins extravascularly. Dicumarol itself or hypoprothrombinemia from any cause appears to have this latter action. Nelson, Castronova, and Hyman (10) demonstrated increased capillary permeability in rabbits following dicumarol and we have observed reduced hematocrits in rabbits on dicumarol (6) and in cholecystnephrostomized dogs (4).

That there is no agreement between the values reported for the one- and two-stage methods is not unusual. Such a discrepancy has been shown to exist between the two methods of assay when they are used to assess clotting efficiency in the newborn, in liver disease, and following indirect anticoagulant administration. In addition, we have noted this phenomenon recently following the administration of a severe chemical stress to rats. It appears that the values for the one-stage are affected more readily than are those of the two-stage.

It is seen from the results reported that while the exposure of dicumarol-treated rats to stress causes some increase in variability and even a slight increase in the mean prothrombin time, a much more dramatic effect is the mortality from hemorrhage which results. Mortality from hemorrhage was even more marked when dicumarol treatment was combined with adrenalectomy. With 10 mg./kg. dicumarol, the adrenalectomized animals had prothrombin times of the same order as normal rats on 20 mg./kg. dicumarol, but the adrenalectomized rats all died, whereas the normal animals all survived.

In hemostasis there are three lines of defense: (1) coagulation of the blood, (2) agglutination of platelets and blood cells, (3) constriction of blood vessels and the vascular resistance. With one of these defenses defective, adjustment can still be made, but, if more are impaired, it is unlikely that hemorrhage can be prevented, and death results. The mortality observed in these experiments when different treatments are combined probably represents the combination of multiple defects in the three lines of hemostasis.

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UPTAKE OF RADIOACTIVE SULPHUR BY VARIOUS TISSUES OF NORMAL AND CHOLESTEROL-FED RABBITS[1]

R. C. Buck and F. C. Heagy[2]

Abstract

The uptake of inorganic sulphur labelled with radioactive sulphate (S^{35}) was studied in rabbits that had been fed a normal diet and rabbits that had been fed a similar diet supplemented by the addition of 1% of cholesterol for 4 months. Paper electrophoresis of alkaline extracts of cartilage, tendon, or aorta separated a peak of radioactivity that corresponded to a band that stained metachromatically with toluidine blue and that is assumed to be chondroitin sulphate. Characteristic peaks of radioactivity were found by electrophoresis of extracts of mucin-containing tissues of the gastrointestinal tract, indicating the location of sulphated mucus with a mobility different from that of chondroitin sulphate. No radioactive material was isolated from the tissues of cholesterol-fed animals that was not present in normal tissues. Highly significant over-all differences in specific activity of sulphur were found between various tissues in both normal and cholesterol-fed rabbits. A significant elevation of both specific activity and sulphur concentration was produced in the aorta as a result of cholesterol feeding.

Introduction

It has been shown by Odeblad and Boström (8), using normal animals, and by Buck (4), using cholesterol-fed animals, that radioactive sulphate (S^{35}) injected into rabbits is incorporated into the aorta. Usually the extent of incorporation parallels the intensity of staining for acid mucopolysaccharide. It was assumed, for this reason, that the principal substance incorporating S^{35} was chondroitin sulphate. The present study was designed to investigate more precisely the identity of the substance or substances taking up S^{35} in normal and cholesterol-fed rabbit aorta. In order to characterize the sulphur-containing material of the aorta it was necessary to compare it with sulphur-containing extracts obtained from other tissues under similar circumstances.

Methods

Animals

The experiments were done with male rabbits weighing 2–3 kg. For each experiment tissues were obtained from four rabbits on a stock diet (Master Feeds) and four rabbits that had been fed this diet supplemented by the addition of 1 % by weight of cholesterol U.S.P. The cholesterol was dissolved in ether and intimately mixed with the pellets. Both the normal diet and cholesterol-supplemented diet were fed for a period of 4 months. The aortas of all cholesterol-fed rabbits showed the characteristic plaques of experimental cholesterol atherosclerosis.

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Carrier-free sulphur^{35} in the form of neutral sodium sulphate was injected into the marginal ear vein in a dose of 650 μc./kg. body weight and 48 hours later the animals were killed by air embolism.

Electrophoresis of Tissue Extracts

The following tissues were studied: thoracic aorta, costal cartilage, tracheal cartilage, tendon, salivary gland, stomach, and small intestine. Weighed amounts of these tissues were placed in 15 ml. centrifuge tubes, to each of which was added approximately twice the tissue volume of 0.5 *N* KOH. After 48 hours at 4° C. the tubes were centrifuged at 1500 r.p.m. for 5 minutes. To 1.0 ml. of the supernatant solution was added sufficient 5% perchloric acid to bring the pH to 8.6. As the acid was added, a precipitate formed. The tubes were centrifuged and the supernatant was used for paper electrophoresis, which was carried out at 4° C. using barbital buffer at pH 8.6. Using an Agla micrometer syringe, 0.1 ml. aliquots of the supernatant were applied to transverse lines 7 cm. from the anodal end of strips of Whatman 3 MM filter paper 72 cm. long by 2.5 in. wide. The papers had been dipped previously in barbital buffer and equilibrated for 1 hour with the current on. A potential of 3.5 volts per cm. was applied for 18 hours. The papers were dried in a vertical position with the aid of heat and forced circulation. The resulting bands of radioactive material were located by autoradiography on Kodak No-screen X-ray film. The density of the autoradiographs was measured on a recording densitometer.

Determination of Total Sulphur and Specific Activity

In another experiment two groups of animals consisting of four normal and four cholesterol-fed rabbits were injected with S^{35} and killed as described above. All cholesterol-fed rabbits had typical aortic lesions visible. The following tissues were grossly removed for study: aorta, liver, kidney, costal cartilage, stomach, and small intestine. The analytical method was a modification of the method of Stockholm and Kock (10). Weighed amounts of these tissues (1–10 g.) were extracted with 20–100 ml. of acetone in a mortar; the supernatant was discarded and the residue dried in an oven at 37° C. To the dry residue was added 10–20 ml. of 10% NaOH which solubilized the residue in 24–48 hours at room temperature. After neutralization with concentrated hydrochloric acid, the solution was acidified by the addition of 4 ml. of concentrated nitric acid and heated in a Kjeldahl flask until salts began to come out. Portions of concentrated nitric acid (4 ml. each) and bromine (20 drops each) were added several times until a clear solution resulted. Water (20 ml.) was added and the solution then evaporated almost to dryness to remove most of the nitric acid. After the addition of another 20 ml. of water, the solution was neutralized with sodium hydroxide and 5 ml. of concentrated hydrochloric acid added. The solution was filtered into a 50 ml. centrifuge tube and washings from the flask added. Sulphate was precipitated in the centrifuge tube by the addition of 5 ml. of 10% barium chloride. The contents were boiled for 10 minutes, allowed to stand for

several days, and centrifuged, and the supernatant was discarded. The precipitate was washed several times with distilled water, centrifuged between washings, and finally suspended in a few milliliters of acetone. The washed precipitate was plated onto a 1 sq. cm. area of a tared aluminum planchette, dried, weighed, and counted in a gas-flow counter (Nuclear Instruments, Chicago). Radioactivities were calculated as counts per minute per square centimeter measured at infinite thickness (23 mg./sq. cm.) after correcting for decay. The resulting values are proportional to specific activity. The concentration of sulphate was expressed as percentage of wet weight of tissue as barium sulphate, determined from the weight of the precipitate on the planchette.

Results

Fig. 1 shows the distribution of density along the autoradiographs from tissues of normal animals and the aorta of a cholesterol-fed rabbit. The location of the peaks is summarized in Table I. There was never any appreciable difference between the normal and cholesterol-fed rabbits in the location of the peaks of activity.

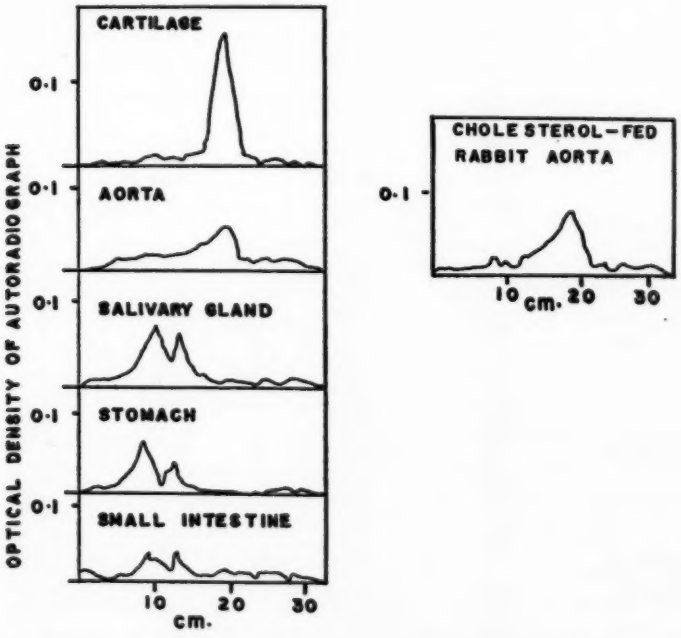

FIG. 1. Tracings of optical density measurements along typical autoradiographs of electropherograms. The tracings on the left represent tissues of normal animals; that on the right represents the aorta of a rabbit after cholesterol feeding. Measurements in centimeters are shown from the starting line on the paper.

TABLE I

LOCATION OF PEAKS OF S^{35} ACTIVITY ON PAPER ELECTROPHEROGRAMS OF ALKALINE EXTRACTS OF VARIOUS RABBIT TISSUES (3.5 VOLTS/CM., 18 HOURS)

Values given in cm. from origin

Costal cartilage	18–20
Tracheal cartilage	18–20
Tendon	18–20
Normal aorta	18–20
Atheromatous aorta	18–20
Salivary gland	7–11 and 12–14
Stomach	7–11 and 12–14
Small intestine	0, 7–11, and 12–14

TABLE II

RADIOACTIVITY AND CONCENTRATION OF SULPHUR ($BaSO_4$ AS % WET WEIGHT) IN VARIOUS TISSUES OF NORMAL AND CHOLESTEROL-FED RABBITS

	Radio-activity*	% of wet wt. $BaSO_4$	Radio-activity*	% wet wt. $BaSO_4$
Tissue	Normal rabbits		Cholesterol-fed rabbits	
Costal cartilage	780	4.28	627	1.03
	924	1.70	903	3.25
	982	1.23	845	1.05
	—	—	705	2.67
Mean ± S.E.M.	895 ± 59	2.40 ± 0.95	770 ± 52	2.00 ± 0.57
Stomach	1890	0.42	1284	0.42
	2020	0.37	3070	0.39
	1970	0.35	1560	0.30
	2022	0.27	2000	0.37
Mean ± S.E.M.	1976 ± 31	0.36 ± 0.04	1979 ± 393	0.37 ± 0.03
Liver	1210	0.59	948	0.65
	1120	0.38	2185	1.30
	1321	0.33	1204	0.51
	935	0.83	1374	0.46
Mean ± S.E.M.	1147 ± 32	0.53 ± 0.16	1428 ± 268	0.73 ± 0.19
Kidney	2100	0.52	1460	0.31
	1820	0.48	2740	0.59
	1780	0.59	1910	0.41
	1860	0.46	1960	0.54
Mean ± S.E.M.	1890 ± 31	0.51 ± 0.04	2018 ± 265	0.46 ± 0.09
Aorta	1550	0.45	2380	0.48
	1140	0.43	5010	0.52
	1560	0.32	2070	0.55
	1440	0.41	3120	0.44
Mean ± S.E.M.	1423 ± 98	0.40 ± 0.03	3145 ± 660	0.50 ± 0.03
Small intestine	5240	0.77	2810	0.48
	6410	0.57	7350	0.47
	6040	0.53	6060	0.35
Mean ± S.E.M.	5780 ± 260	0.59 ± 0.08	5045 ± 1020	0.48 ± 0.03

* Counts per minute per square centimeter of $BaSO_4$ at infinite thickness.

Table II gives the radioactivity of sulphur and the concentration of sulphur, expressed as barium sulphate, in the tissues of each animal. Duplicate analyses could not be carried out because of the small size of some of the samples. By the method of analysis of variance the values for specific activity in the normal group show a highly significant over-all between-tissue difference (F ratio* = 185.4). Cartilage has the lowest specific activity and intestine the highest, liver, kidney, and aorta having intermediate values. The concentration of sulphur did not differ significantly in the tissues studied, with the exception of cartilage where it showed a highly significant elevation over that in the other tissues.

Except for aorta the tissues of the cholesterol-fed group showed no significant difference from the corresponding values in the normal group in specific activity or in concentration of sulphur. Compared with the normal group, there was a greater variation between animals, particularly for the specific activity. As with the normal group, the cholesterol-fed group showed a highly significant over-all between-tissue difference in specific activity.

In contrast to the other tissues, the aortas of the normal and the cholesterol-fed groups showed a significant difference (P = .05) in both specific activity and concentration of sulphur. On the average, the specific activity was more than twice as high after cholesterol feeding and the sulphur concentration was increased by 25%. As might be expected, because of the variable degree of the atheromatous state of the arteries, the values for specific activity showed great variation, while those for the normal vessels were relatively uniform.

Discussion

Extracts of mucus-producing epithelial tissues (salivary gland, stomach, and intestine) and connective tissues (cartilage and aorta) were chosen so that the chondroitin sulphate might be compared with sulphated mucus in regard to electrophoretic mobility, radioactivity, and sulphate concentration. Obviously, on the basis of electrophoresis they may be distinguished easily, the mucus-producing tissues having two characteristic peaks of lower mobility than the single peak of the connective tissues.

It has been observed both in this study and by other workers that autoradiographs of intestine show pronounced uptake of sulphate by the epithelium. Boström and Odeblad (3), in a study by autoradiography of the time sequence of S^{35} uptake in intestine, noted two distinct sulphate fractions, distinguishable on the basis of their biological half-life. The first of these, with a biological half-life of 5 days, was located in the goblet cells and produced very dense and circumscribed autoradiographs. The second was more diffusely distributed and produced a fainter autoradiograph, but had a longer biological half-life, a week or so. The latter appeared in association with mucigen granules in epithelial lining cells. In view of these findings it is tempting to speculate that the two bands of activity we have observed may represent

* Ratio of the between-tissue variance to the within-tissue variance.

mucus from goblet cells in one and epithelial mucigen granules in the other. On the other hand, Bélanger (1), who was able to observe the uptake of S^{35} by mucous neck cells of stomach as early as an hour after injection, did not find S^{35} incorporation into the mucigen granules of the lining epithelial cells. This was confirmed by Jennings and Florey (6), who observed that the foveolar cells also showed S^{35} uptake. Moreover, in the salivary gland, the great preponderance of mucus is produced by the mucous alveolar cells (although a few goblet cells are found in the ducts). Yet in both stomach and salivary gland extracts two bands are observed on electrophoresis in the same location as in the intestine extracts. It seems likely that by electrophoresis of alkaline extracts we have isolated two separate fractions, not related to any particular cell type or anatomic structure, but characteristic of mucus of the gastrointestinal tract generally.

Recently, in a study of S^{35} incorporation into duodenal juice of rabbits, Kent, Whitehouse, Jennings, and Florey (7) have found a very rapid appearance of injected S^{35} in mucus. By dialysis they succeeded in isolating two fractions from the unhydrolyzed mucus—one which was not dialyzable and did not move on electrophoresis and a second which was dialyzable. They state that the latter is a sulphate ester and they were unable to obtain conclusive results on its electrophoretic mobility.

Electropherograms of cartilage, tendon, and aorta all showed a single peak 18–20 cm. from the origin. Boström and Gardell (2) also observed a single peak of radioactivity on electrophoresis of extracts of rat skins. By staining the paper strips with toluidine blue solution a metachromatic band 18–20 cm. from the origin could be demonstrated in electropherograms of cartilage and could be seen consistently in the strips carrying extracts of tendon or aorta when a large volume (1.0 ml.) of extract was used.

The finding of low specific activity of sulphur in cartilage is not surprising in view of the low rate of metabolism of this tissue. The high concentration of sulphur^{35} in cartilage cells was confirmed by autoradiographs and was observed previously by Pelc and Glucksmann (9). This is compatible with the low specific activity, since the concentration of sulphur was several times higher than in other tissues.

The finding of a higher specific activity of sulphur in the atheromatous aorta extends the impression previously obtained from autoradiographic evidence (4). Here it was felt that the specific activity of sulphur in the intimal lesion was higher than the normal value and that in the media was probably lower. Apparently the net result, which we have now determined, is an increased specific activity.

The observation of an elevated sulphur concentration in cholesterol atherosclerosis of rabbits agrees with the finding by Faber (5), who studied human atherosclerosis.

Acknowledgment

The authors express their appreciation to Mr. William Daniels for technical assistance.

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THE ESTIMATION OF MALEIC HYDRAZIDE BY PAPER CHROMATOGRAPHY[1]

W. A. ANDREAE

Abstract

Maleic hydrazide added to solutions of partially purified plant extracts can be determined semiquantitatively by a paper chromatographic method in which the area of the maleic hydrazide spot, detected by spraying with a mixture of ferric chloride and potassium ferricyanide, is measured. By this method as little as 0.2 micrograms of maleic hydrazide can be detected with an error of about 35%. This method is not applicable to plants treated with maleic hydrazide because of the presence of interfering substances.

Simple and convenient methods for the determination of maleic hydrazide are needed for studies on the mechanism of inhibition of plant growth by this substance. The present paper deals with an application of paper chromatography to this problem.

Maleic hydrazide, which exists predominantly in the monoenolic 6-hydroxy-3 (2H)pyridazinone form (5), was found to give a color reaction with a number of phenol reagents. Of those tested, a freshly prepared 1:1 mixture of aqueous 1% ferric chloride and aqueous 1% potassium ferricyanide, introduced by Barton *et al.* (3), gave the most clearly defined spots with maleic hydrazide on paper chromatograms. While most phenolic compounds give an instantaneous blue color with this reagent, the color does not develop for several minutes with maleic hydrazide. With time, the background of the chromatogram also turns dark blue so that after an hour much of the contrast between spot and background is lost. Indoleacetic acid (4) and tryptophan also give a color reaction with the ferric chloride – potassium ferricyanide reagent.

A satisfactory displacement of maleic hydrazide giving well defined spots was obtained with solvent mixtures of either *n*-butyl alcohol – acetic acid – water (5:1:4) or isopropyl alcohol – concentrated ammonium hydroxide – water (70:10:20). With the *n*-butyl alcohol – acetic acid solvent, maleic hydrazide has an R_f of 0.59 and with the isopropyl alcohol – ammonium hydroxide solvent an R_f of 0.38. Increasing the isopropyl alcohol content from 70% to 80% decreases the R_f to 0.18 while decreasing the isopropyl alcohol content to 60% increases the R_f to 0.56. In the absence of ammonium hydroxide the spots are poorly defined; increasing the ammonium hydroxide concentration beyond 10% has no further effect on the shape of the maleic hydrazide spot.

To determine the concentration of maleic hydrazide in solution, 10 microliter samples are applied 2.5 cm. from the bottom of a 16 × 40 cm. Whatman No. 1 filter paper and chromatographed by the ascending technique in either

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Contribution No. 109, Science Service Laboratory, Canada Department of Agriculture, London, Ontario.

of the partitioning solvents described above. After about 18 hours the paper is dried and sprayed with a freshly prepared 1:1 mixture of aqueous 1% ferric chloride and aqueous 1% potassium ferricyanide. Ten minutes after the paper is sprayed, the outlines of the colored areas are traced on a transparent sheet of celluloid. The spots are cut out of the celluloid and weighed. From a celluloid sheet the ratio of weight to area can be established and the spot weights converted to square centimeters of spot area. With solutions of 0.21 to 4.64 μg. of maleic hydrazide the spot area increases directly with the logarithm of the concentration of maleic hydrazide applied as shown in Fig. 1. The means of quadruplicate determinations on solutions of varying maleic hydrazide concentrations fall on a straight line. By means of this calibration curve it is possible to estimate the concentration of maleic hydrazide with an error of about 35%. Indoleacetic acid can also be estimated with the same degree of accuracy by the same procedure.

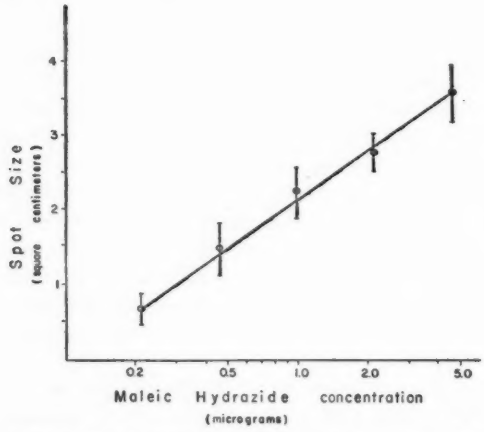

FIG. 1. The relation of maleic hydrazide concentration to spot size. Aqueous solutions were applied as 10 μl. aliquots containing 0.21; 0.46; 1.00; 2.16; or 4.64 μg. of maleic hydrazide and chromotographed as outlined in the text. The vertical lines represent twice the standard error of the mean of quadruplicate determinations.

Maleic hydrazide can be determined in partially purified tissue extracts, such as indoleacetic acid oxidase preparations, without preliminary purification. A typical chromatogram on the determination of maleic hydrazide in the presence of indoleacetic acid oxidase and indoleacetic acid is shown in Fig. 2. In addition to the maleic hydrazide and the indoleacetic acid spots, a small spot due to an unidentified compound in the oxidase preparation is found below the maleic hydrazide spot. The concentration of maleic hydrazide and of indoleacetic acid estimated from chromatograms shows that, while maleic hydrazide triples the rate of enzymatic oxidation of indoleacetic acid as previously reported (2), maleic hydrazide is not affected in the process nor does the maleic hydrazide content decrease on incubation with the oxidase in

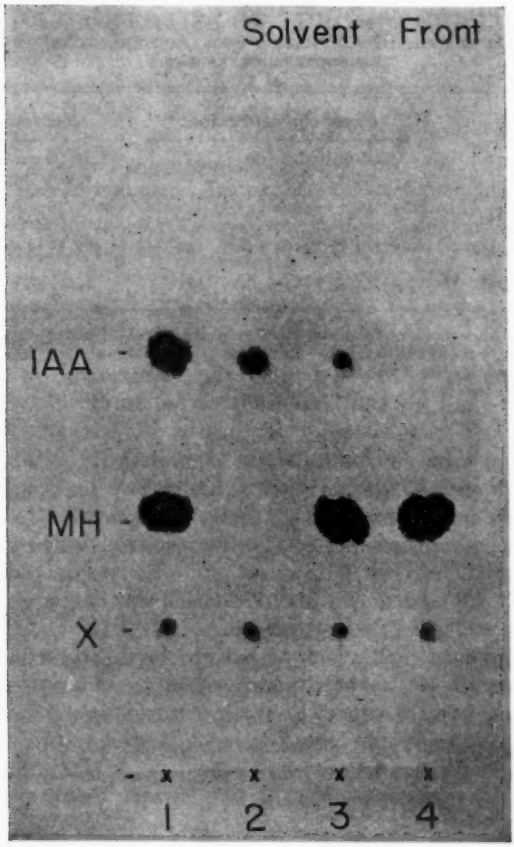

FIG. 2. Chromatogram of maleic hydrazide and indoleacetic acid in the presence of indoleacetic acid oxidase preparation. All four test solutions contained 2.5 ml. of water, 2.5 ml. indoleacetic acid oxidase prepared by the method (2) and, when indicated, 0.5 mg. indoleacetic acid and/or 1 mg. maleic hydrazide. Ten-microliter aliquots were chromatographed as outlined in the text: (1) indoleacetic acid and maleic hydrazide before incubation; (2) indoleacetic acid alone after 60 minutes' incubation; (3) indoleacetic acid and maleic hydrazide after 60 minutes' incubation; (4) maleic hydrazide alone after 60 minutes' incubation.

absence of indoleacetic acid. This suggests that maleic hydrazide, unlike monohydric phenols such as 2,4-dichlorophenol (6), does not act as an oxidation–reduction system in the oxidation of indoleacetic acid.

While this method lacks the sensitivity of the commonly used method of Wood (7), in which the hydrazine produced on reduction and hydrolysis of maleic hydrazide is measured, it has the advantage, apart from simplicity, of detecting maleic hydrazide directly. That the hydrazine content does not necessarily represent only unaltered maleic hydrazide can be seen from a

TABLE I

PHOTOOXIDATION OF MALEIC HYDRAZIDE EVALUATED BY TWO METHODS OF ANALYSIS

Expt. No.	Maleic hydrazide added, mg.	Paper chromatography*		Hydrazine content†	
		Maleic hydrazide found, mg.	Loss, %	Maleic hydrazide found, mg.	Loss, %
1	2.00	0.60	70	1.50	25
2	2.00	0.63	69	1.35	32
3	2.00	0.97	51	1.54	23

NOTE: Solutions also contained 0.3 mg. riboflavin per 10 ml. of water and were adjusted to pH 7.0. Solutions were illuminated by 1000 foot-candle incandescent light for 60 minutes with gentle shaking in an ice bath.

* By the method described.

† By the method of Wood (7).

comparison of the results of studies on the photooxidation of maleic hydrazide by the two methods. While the results obtained by paper chromatography (Table I) indicate extensive photooxidation of maleic hydrazide in the presence of riboflavin, as previously reported (1), the results obtained from hydrazine analysis show a relatively small decrease. In other words, the major part of the maleic hydrazide undergoing photooxidation was degraded only as far as a product which still gives rise to hydrazine.

The paper chromatographic method has not been satisfactorily applied to intact tissues treated with maleic hydrazide because of the presence of interfering substances. So far efforts to separate such phenolic interfering substances from maleic hydrazide have been unsuccessful and for tissue analysis the method of Wood (7) is recommended.

The author wishes to thank Mr. M. W. H. van Ysselstein for technical assistance.

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THE PROPERTIES OF THROMBINOGEN[1, 2]

EDWARD RONWIN

Abstract

Evidence is given which demonstrates the existence of an intermediate, designated *thrombinogen*, during the conversion of prothrombin to thrombin. The conversion of prothrombin to thrombinogen requires thromboplastin. The conversion of thrombinogen to thrombin requires thrombin, though other tryptic enzymes can replace thrombin. The properties of thrombinogen have been studied.

Introduction

As a result of observations on the properties of thrombin (3, 4), the author suggested that an intermediate, designated *thrombinogen*, is formed during the conversion of prothrombin to thrombin. Shulman (5) and Spaet (6), on independent grounds, also recognized the inadequacy of a simple conversion of PT to T. The validity of the proposal has now been demonstrated. As a guide to isolation attempts, the studies reported here were undertaken to determine some of the chemical and physical characteristics of this intermediate.

Materials and Methods

Standard Conditions and Measurement of Enzyme Activity

Thrombinogen was measured by gauging its conversion to thrombin, which is, in turn, determined by its action on TAME. The test methods, therefore, are those previously used with T (3) and the description of standard conditions is identical. Again, the conditions themselves are: initial substrate concentration, 0.01 *M* TAME (only synthetic substrate used); temperature, 38° C.; pH, 8.0 ± 0.05; buffer, 0.015 *M* Veronal; ionic strength, 0.025; reaction volume, 2 ml.

The Th Unit of Thrombin Activity

The Th unit of thrombin activity used herein has been fully described (3).

Proteins and Substrates Employed

Thrombin, topical (Parke, Davis, & Co.), lot No. 029342 was used throughout. The prothrombin was prepared as described by Lewis and Ware (2), except that the ammonium sulphate precipitation steps were not included.

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Contribution from the Department of Pharmacology, University of Southern California, School of Medicine, Los Angeles 7, Calif. Present address: Department of Biochemistry, University of Alabama Medical Center, Birmingham 3, Alabama.

[2]Abbreviations in text and tables: TRB — thrombinogen; PT — prothrombin; ThP — thromboplastin; T — thrombin; F — fibrinogen; TAME — N^{α}-*p*-toluenesulphonyl-L-arginine methyl ester. When brackets are placed about two or more of these abbreviations, it indicates that the elements within the brackets were treated together in the manner described. While [PT + ThP] and TRB are equivalent, the choice of abbreviation was dictated by the meaning intended to be conveyed.

This procedure yields a very satisfactory product and the aliquots of the prothrombin preparation refer to the aliquots of the solution obtained using this procedure. Fifty milliliters of prothrombin preparation in 0.225 *M* NaCl plus 0.02 *M* citrate were obtained from 100 ml. of human plasma. The ThP preparation was an isotonic saline extract of human brain made available by Dr. A. G. Ware.

TRB solution resulting from the combination of PT and ThP was prepared free of salts by dialysis. Twenty milliliters of TRB solution was dialyzed against three changes of 4 liters of distilled water during a 24 hour period. The TRB solution that resulted was 20 ml. in volume and free of chloride ion.

The fibrinogen used was a 0.1% solution in 0.3 *M* KCl and was made available by Dr. A. G. Ware. TAME was a commercial product of H & M Chem. Co., Santa Monica, Calif.

Results and Discussion

Evidence for the Existence of Thrombinogen

Table I presents the evidence which attests to the validity of the proposal of the existence of TRB. When PT and T are incubated, no excess activity is observed (line 4). Similarly, ThP alone has no TAME-splitting activity (line 5), nor does it affect the action of T in the presence of salts (line 6).

TABLE I

DEMONSTRATION OF THROMBIN GENERATION FROM THROMBINOGEN AS MEASURED BY TAME HYDROLYSIS

No.	T	ThP	PT	0.01 *M* Na_3 citrate	0.133 *M* NaCl	0.015 *M* $CaCl_2$	% hydrolysis of TAME at 5 min.	Relative rate*
1	×						28.5	
2	×			×	×		17.6	
3	×			×	×	×	23.8	
4	×		×	×	×		17.7	1.00
5		×					0.0	
6	×	×		×	×	×	23.8	1.00
7		×	×	×	×		0.0	
8		×	×	×	×	×	0.0	
9	×	×	×	×	×		28.5	1.62
10	×	×	×	×	×	×	30.1	1.27
11	×	×	×†	×	×		19.0	1.08
12	×	×	×‡				39.8	1.40
13	×	(trypsin)					41.2	
14	×			×	×		36.4	
15	×	×	×	×	×		42.8	1.18

* TRB conversions and tests of enzymatic activity are mutually inclusive and were run under standard conditions plus salts as indicated; PT conc.: 0.5 ml. of the preparation; ThP conc.: 0.1 ml. of the preparation. The relative rate is the ratio of the percentage hydrolysis of the test under consideration to the base run; for thrombin: runs containing NaCl and citrate are compared to line 2; those runs containing additionally $CaCl_2$ are compared to line 3.

† Kept 15 minutes in boiling water bath prior to use.

‡ [PT + ThP] solution dialyzed to remove salts. TRB present was equivalent to one-half that in the other runs. Run is compared to line 1.

When PT and ThP are incubated together no activity is observed (line 7); neither does the addition of calcium chloride produce activity (line 8). However, when PT, ThP, and T are incubated together, a 62% increase in TAME hydrolysis is observed within 5 minutes of reaction (line 9). Thus, T cannot release T from PT unless PT and ThP have first interacted, either by complex formation or by the enzymatic action of ThP on PT. In either case, a third entity (TRB) is formed which is different from its precursor PT and capable of being converted to T by T. Line 10 illustrates the over-all inhibitory action of calcium chloride on the PT conversion; however, as Ca^{++} is a known activator within limits of T (3), its inhibitory action may be on the conversion of PT to TRB. Heat treatment of PT resulted in almost complete inactivation of PT, virtually eliminating its conversion to T (line 11) and shows that the basic source of the additional TAME-splitting activity is PT. When [PT + ThP] is dialyzed to remove salts, activity increases (line 12). This result also demonstrates that neither sodium chloride, sodium citrate, nor Ca^{++} ion is necessary for the conversion process to proceed. (The dialyzed [PT + ThP] possessed no TAME-splitting activity alone.)

Lines 13 to 15 demonstrate that trypsin can also convert TRB to T. This is supported by the observations of others (1) that trypsin accelerates blood clotting. Since the specificities of T, plasmin, and trypsin are so alike (3, 4), the conversion process is possibly due, in part, to the hydrolysis of a peptide bond in which the C=O group is donated by an arginyl or lysyl moiety. Also, the similarity between the systems thrombinogen–thrombin and trypsinogen–trypsin is suggestive.

The data of Table II should dispel argument that the increased TAME-splitting activity may be due to a "tryptic" principle other than T. To obtain the data of line 1, Table II, PT + ThP were incubated together while T was incubated separately. After a 20 minute period the addition of the

TABLE II

DEMONSTRATION OF THROMBIN GENERATION FROM THROMBINOGEN AS MEASURED BY FIBRINOGEN CLOTTING

	Reaction mixture	Clotting time (seconds)
1.	[PT + ThP] (0.2 ml.)* + T (0.2 ml.)* + F (0.3 ml.)†	301
2.	[PT + ThP + T] (0.2 ml.)* + salt solution to give equal concentrations in each test (0.2 ml.)* + F (0.3 ml.)†	167

* Incubation conditions prior to clotting test: 0.133 *M* NaCl; 0.01 *M* Na_3 citrate; 38° C.; pH 8; 0.015 *M* Veronal buffer.
† 0.1% fibrinogen solution (F) in 0.3 *M* KCl.

components was made in rapid succession in the order given. In case 2, PT + ThP + T were incubated together at the same concentrations as in case 1 prior to the clotting test. [PT + ThP] was inactive; nor did Ca^{++} addition produce clotting. T alone had a clotting time about equal to that of case 1.

The results show that where T is allowed to act upon the product of the interaction of ThP and PT, additional enzyme activity arises which evidences itself both by TAME hydrolysis and by clotting action, the hallmarks of T activity. Consequently, the process of PT conversion to T, which for the last 60-odd years has been generally depicted as follows (1):

$$[1] \qquad PT \xrightarrow[Ca^{++}]{ThP} T$$

is now more properly written:

$$[2] \qquad PT \xrightarrow{ThP} TRB \xrightarrow{T} T.$$

The data presented here support the additional view that Ca^{++} ion is unnecessary for the conversion of prothrombin to thrombin.

Effects of Time of Incubation of PT and ThP on TRB Formation

The data are given in Table III. After 5 minutes of incubation and for at least 10 minutes thereafter, the yield of TRB is constant. In all experiments that followed, a 5 minute interval was used prior to T addition. The data also show that the yield of TRB is essentially unaffected by the presence or absence of TAME during the incubation.

TABLE III

EFFECT OF TIME OF INCUBATION OF PROTHROMBIN PLUS THROMBOPLASTIN ON THROMBINOGEN FORMATION

Incubation time (min.)*	% hydrolysis of TAME in 10 minutes†	Relative rate
	Incubation in the presence of TAME‡	
	28.5§	1.00
0	38.0	1.33
5	45.3	1.59
10	43.8	1.54
15	45.6	1.60
	Incubation in the absence of TAME‖	
5	45.2	1.58
15	42.8	1.50

* For the case of incubation in the presence of TAME: time elapsed between addition of ThP and enzyme addition. For the case of incubation in the absence of TAME: time of [ThP + PT] incubation at the end of which substrate and enzyme were added in rapid succession.

† TRB conversions and tests of enzymatic activity are mutually inclusive and were run under standard conditions plus 0.133 *M* NaCl and 0.01 *M* Na_3 citrate; PT conc.: 0.5 ml. of the preparation; ThP conc.: 0.1 ml. of the preparation; original thrombin concn.: 3.0 Th units/ml.

‡ During the incubation everything was present except enzyme.

§ Value in the absence of [PT + ThP].

‖ During the incubation everything was present except substrate and enzyme.

Stability Studies

PT and ThP were incubated together for 5 minutes to permit maximum TRB formation. The mixtures were then subjected to 65° C. at pH 2.2 for 20 minutes and to 95° C., at pH 8 for 1 hour in the presence, in both cases, of 0.01 *M* sodium citrate and 0.133 *M* sodium chloride. In neither case was the capacity of TRB to be converted to T reduced.

Relationship between PT Concentration and TRB Generation

The data of Fig. 1 show that the generation of TRB under the conditions and concentration range employed bears a straight-line relationship to PT concentration.

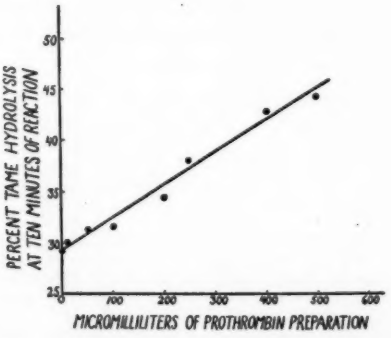

FIG. 1. Effect of prothrombin concentration on thrombinogen generation. TRB conversions and tests of enzyme activity are mutually inclusive and were run under standard conditions plus 0.133 *M* NaCl and 0.01*M* Na_3 citrate; ThP conc.: 0.01 ml. of the preparation; original thrombin concn.: 3.01 Th units/ml. [PT + ThP] incubation for 5 minutes prior to T addition.

pH Optimum of TRB Generation

As shown above, TRB is formed by the interaction of PT and ThP. The subsequent conversion of TRB to T is mediated by T. Each step of the reaction (equation [2]) presumably has a pH optimum. The data of Fig. 2 present the over-all optimum of the reaction in the presence of sodium chloride and citrate, while those of Fig. 3 present the pH optimum only of the TRB conversion to T in the absence of these salts. Curve A in Fig. 2 represents the sum of T activity generated by TRB conversion plus the initial T activity added to the system containing [PT + ThP]. Curve B, Fig. 2, represents the blank which is simply the curve for the action of T on TAME in the same concentrations as used to obtain Curve A. Subtraction of Curve B from Curve A yields Curve C, which represents (or is proportional at all points to) the T activity generated from TRB as a function of pH. Under the conditions prevalent in Fig. 2, the pH optimum of the over-all process appears to be quite broad ranging from pH 7–9. If the data are treated in another manner, as presented in Curve D, an interesting result appears. Curve D is

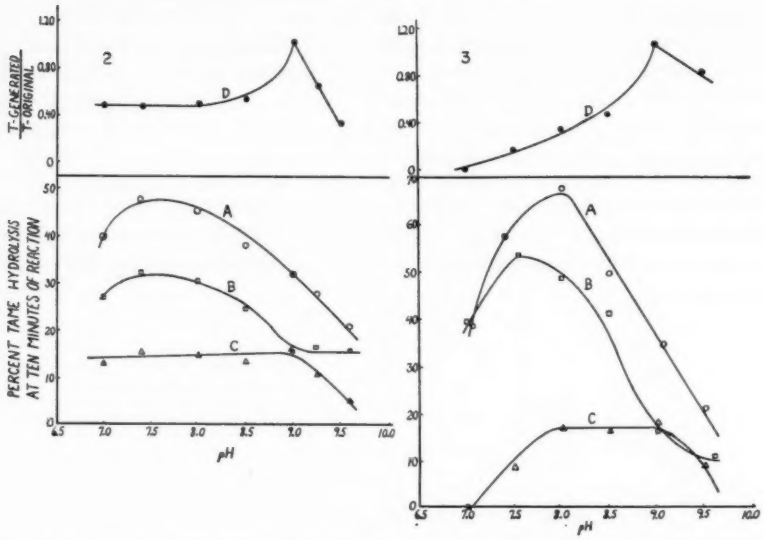

FIG. 2. pH optimum of over-all conversion of prothrombin to thrombin in the presence of NaCl and citrate. TRB conversions and tests of enzymatic activity are mutually inclusive and were run under standard conditions plus 0.133 *M* NaCl and 0.01 *M* Na_3 citrate; pH as indicated. PT conc.: 0.5 ml. of the preparation; ThP conc.: 0.1 ml. of the preparation; original thrombin concn.: 2.97 Th units/ml. [PT + ThP] incubated for 5 minutes prior to T addition; no thrombin activity observed until T addition. Curve A—all entities present; Curve B— [PT + ThP] absent; Curve C — constructed from the values obtained by subtraction of curve B from curve A; Curve D — ratio of Curve C values/Curve B values.

FIG. 3. pH optimum of thrombinogen conversion to thrombin in the absence of salts. TRB conversions and tests of enzymatic activity are mutually inclusive and were run under standard conditions except pH as indicated. TRB conc.: 0.25 ml. of the preparation; original thrombin concn.: 3.06 Th units/ml. Curve A — all entities present; Curve B — TRB absent; Curve C — constructed from the values obtained by subtraction of Curve B from Curve A; Curve D — ratio of Curve C values/Curve B values.

obtained by plotting the ratio of Curve C values/Curve B values. This yields a picture of the relative quantity of T generated (or TRB converted) in the over-all reaction to that of the activity of the initially added T (as judged by TAME-splitting action) over the pH range investigated. It is now seen that the relative amount of T generated (or TRB converted) in the over-all process reaches a sharp maximum in the vicinity of pH 9; although the absolute amount of T produced at this pH is no higher than at any other point in the pH 7–9 region (Curve C).

For further study a quantity of PT was converted to TRB, using ThP, and the resulting TRB was subjected to dialysis. Assuming the absence of a reversible equilibrium between PT and TRB, this enabled a determination of the pH optimum of step (2), equation [2]. Fig. 3 contains the data. Curve A again represents the sum of T activity generated plus the initial T activity added to the system containing TRB. Curve B represents the action of the

original T activity on TAME in the absence of TRB. Subtraction of Curve B from Curve A yields Curve C as the pH optimum curve for the TRB to T conversion step. The amount of T generated is now somewhat more pH dependent than when PT and ThP were allowed to interact during the test period since the optimum range of activation of the TRB narrows to the region of pH 8–9.

Of much additional import is the striking fact that a plot of the ratio of the amount of T generated (or TRB converted) (Curve C) to that of the blank (initially added T activity as judged by TAME hydrolysis) (Curve B) again yields a curve with a peak at pH 9; although the absolute amount of T produced at this pH is no higher than at any other point in the pH 8–9 region. Effectively then, as judged by TAME hydrolysis, a quantity of T acting at pH 9 is less in terms of activity than an equal quantity of T acting at pH 8

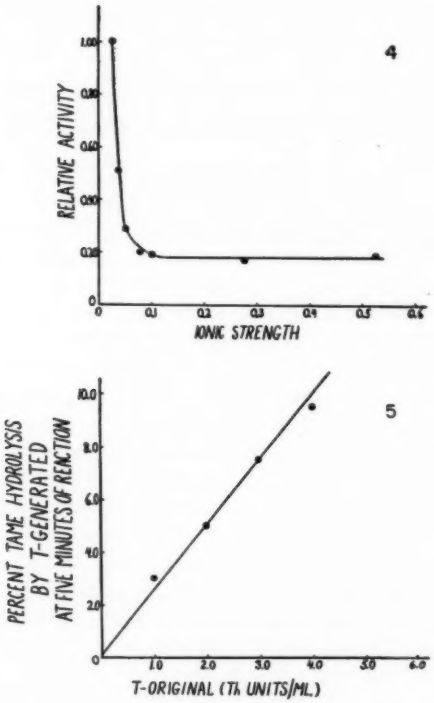

FIG. 4. Effect of ionic strength on thrombinogen conversion to thrombin. NaCl used as ionic strength agent. TRB conversions and tests of enzymatic activity are mutually inclusive and were run under standard conditions except ionic strength. TRB conc.: 0.25 ml. of the preparation; original thrombin concn.: 3.06 Th units/ml.

FIG. 5. Dependence of thrombinogen conversion on thrombin concentration. TRB conversions and tests of enzymatic activity are mutually inclusive and were run under standard conditions. TRB conc.: 0.25 ml. of the preparation. Original thrombin activities as given in the abscissa.

(Curves B, Figs. 2 and 3); yet this same quantity of T is capable of converting a like amount of TRB to T at either pH (Curve C). Hence it takes "less" T (in terms of its TAME-splitting activity) to convert equal quantities of TRB to T at pH 9 than at pH 8. In this sense the pH optimum for TRB conversion is at pH 9, while the pH optimum for TAME hydrolysis is at pH 8 ((3) and Curves B, Figs. 2 and 3).

Effect of Ionic Strength on TRB Conversion

The effect of ionic strength on TRB conversion is shown in Fig. 4. The initial drop in activity is quite steep and is followed by a gradual decrease to a fairly constant value of 20% remaining activity.

Relation Between T Concentration and TRB Conversion

Fig. 5 demonstrates that the conversion of TRB is proportional to T concentration over the range investigated and, as with TAME, is related by a straight-line function. The data imply that the action of T in TRB conversion is similar to T action on TAME.

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OXIDATIVE METABOLISM OF CARBOHYDRATES IN INSECTS

III. HEXOSE MONOPHOSPHATE OXIDATIVE CYCLE IN THE HOUSEFLY, MUSCA DOMESTICA L.[1]

W. CHEFURKA

Abstract

The component reactions of the hexose monophosphate oxidative pathway have been studied in adult houseflies. It has been shown that houseflies possess an active glucose-6-phosphate dehydrogenase, 6-phosphogluconolactonase, 6-phosphogluconic dehydrogenase, phosphopentose isomerase, phosphoketopentose epimerase, transketolase, and transaldolase.

The cycle consists of five main steps: (*a*) a two-step oxidation of glucose-6-phosphate to ribulose-5-phosphate and CO_2; (*b*) isomerization of ribulose-5-phosphate to ribose-5-phosphate; (*c*) epimerization of ribulose-5-phosphate to xylulose-5-phosphate; (*d*) conversion of pentose phosphate to sedoheptulose phosphate and triose phosphate; (*e*) conversion of sedoheptulose phosphate to hexose phosphate.

Balance studies indicate that 2 moles of pentose phosphate are utilized for the formation of 1 mole of heptulose phosphate. However, this stoichiometry is attained after a lag period, suggesting the formation of a transient intermediate. Because the lag was eliminated by the addition of hydroxypyruvate, it is suggested that the lag was due to the time required for the combination of a C_2 fragment with an appropriate aldehyde acceptor.

Introduction

It is now recognized that carbohydrates may be dissimilated in insects by several routes. Glycolysis has been demonstrated in several species (5, 9, 30, 31, 38) but the evidence in many instances lacks the stoichiometry that has been obtained for mammalian tissue. Zebe (55) has reported that the rate-limiting step of insect muscle glycolysis may be lactic dehydrogenase. The tricarboxylic acid cycle has been shown to be localized predominantly on insect mitochondria (38, 43, 44, 45, 50, 54), but it remained for Hoskins *et al.* (29) to demonstrate that, in the presence of EDTA,[2] *theoretical* oxidation of most intermediates could be obtained with honey bee mitochondria. More recently the studies of Newburgh and Cheldelin (41) and studies in this laboratory (10) have revealed the operation of the hexose monophosphate oxidative cycle in certain insects.

The work of Newburgh and Cheldelin (41) and in our laboratory (10) has indicated the presence of aldopentoses, ketopentoses, and heptoses in aphids and houseflies, and further studies in this laboratory (11) have indicated that the properties of G-6-P and 6-PG dehydrogenases in the flight muscles of

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[2] The following abbreviations will be used: EDTA, ethylenediamine tetraacetate; HMP, hexose monophosphate; G-6-P, glucose-6-phosphate; 6-PG, 6-phosphogluconate; TPN, triphosphopyridine nucleotide; TPNH, reduced triphosphopyridine nucleotide; R-5-P, ribose-5-phosphate; Ru-5-P, ribulose-5-phosphate; ThPP, thiamine pyrophosphate; F-6-P, fructose-6-phosphate; S-7-P, sedoheptulose-7-phosphate; G-3-P, glyceraldehyde-3-phosphate; E-4-P, erythrose-4-phosphate; Xu-5-P, xylulose-5-phosphate; 6-PGL, 6-phosphogluconolactone; GA, glycolaldehyde.

houseflies are similar to the G-6-P and 6-PG dehydrogenases occurring in other organisms. However, a direct comparison of the HMP cycle between insects and mammals is as yet impossible because the evidence to date is scanty and does not furnish quantitative data of the type available for mammalian tissue.

The present communication deals with the individual reaction patterns of the HMP cycle in the adult housefly, *Musca domestica* L. A brief account of this work has been published elsewhere (12).

Methods and Materials

Reagents

6-PG was prepared according to Seegmiller and Horecker (47). A sample of Ru-5-P was kindly supplied by Dr. B. L. Horecker. G-6-P and R-5-P were purchased from Sigma Chemical Company. A quantity of sedoheptulosan was kindly provided by Dr. M. Calvin and Dr. N. K. Richtmyer. TPN was purchased from Pabst Laboratories and Sigma Chemical Company. Gluconolactone was obtained from Nutritional Biochemicals Corporation. D-Ribulose was prepared from D-arabinose by the method of Glatthaar and Reichstein (17) and D-xylulose from D-xylose by the method of Schmidt and Treiber (46). Hydroxypyruvate was generously provided by Dr. G. D. Thorn. All barium salts were converted to the sodium salts by treatment with a slight excess of sodium sulphate.

6-PG dehydrogenase was prepared according to Horecker and Smyrniotis (23). Lactic dehydrogenase and G-6-P dehydrogenase were purchased from Nutritional Biochemicals Corporation. This preparation of lactic dehydrogenase was found to be free of pentose isomerase activity and contributed only 0.3% to the 6-PG dehydrogenase activity of the insect preparation. Acid phosphatase was prepared according to Calvin* or Hochster (21). It possessed some phosphopentose isomerase activity but since this preparation was added to an incubation mixture where an equilibrium between the aldo- and keto-pentoses had already been achieved, no interference from this impurity would be expected. This was confirmed by measuring the level of ketopentoses before and after dephosphorylation; no change in the level of ketopentoses was detected. When equilibrium was not achieved this isomerase activity was corrected for in appropriate blanks. A 1 milligram quantity of the acid phosphatase prepared according to Calvin* hydrolyzed 1.9 μM. R-5-P per hour at pH 5.0 and 35° C.

Insects

The insects used in this study were adult houseflies, *M. domestica* L. They were taken from a laboratory culture maintained according to Fisher (16). The houseflies were starved by withholding all food but water from the moment of adult emergence.

* Calvin, M. Private communication.

Assay Methods

Spectrophotometric measurements were made at room temperature with a Beckman Model DU spectrophotometer.

The pentoses were determined according to Mejbaum (40) with 40-minute heating using a standard calibration curve or the extinction coefficient given by Newburgh and Cheldelin (41). Where necessary, the pentose values were corrected for interference by heptoses. The heptoses and hexoses were assayed with the sulphuric acid – cysteine method (15) after 18 hours, using either a standard curve or the molar extinction coefficient as given by Newburgh and Cheldelin (41). Ketopentose phosphates were determined according to Bruns (8) with a 40 minute hydrolysis at 60° C. and a molar extinction coefficient of 30,000 (49). Free ketopentoses were determined by the cysteine–carbazole method (14) after 2 hours, using the appropriate extinction coefficient. Triose-P was measured as the alkali-labile phosphate using the method of Allen (1). Hestrin's modification (20) of the hydroxamic acid method of Lipmann and Tuttle (36) was used for determinations of 6-phosphogluconolactone. The hydrolysis of the lactone was determined by the hydroxamic acid method using phosphate buffered hydroxylamine at pH 7.5. Protein was determined by the method of Lowry *et al.* (37). 6-PG was determined by the method of Horecker and Smyrniotis (23).

Paper Chromatography

Ascending chromatograms were made on Whatman No. 1 filter paper with 77% acetone and acetone–water (10 : 3 v/v) saturated with boric acid. The latter solvent permitted a good separation of aldopentose from ketopentose. The pentoses were located by spraying with aniline hydrogen phthalate (42), TCA–orcinol (33), or cysteine–carbazole (49) with heating for 5 minutes at 100° C. The latter two sprays were particularly useful in locating the ketopentoses on chromatograms run in 77% acetone. Controls of the appropriate sugars were always run with the unknowns.

Column Chromatography

This was done on Dowex-1 resin (Dow Chemical Co., Midland, Michigan; 200 mesh, 10% cross linked) in borate form for free sugars (35). Analytical columns, 1 cm. diameter × 12 cm. were used. Fractions of 11 ml. at a flow rate of 0.5 ml. per minute were received in an automatic collector and analyzed by the methods described. The free ketopentoses were eluted with 0.02 *M* sodium borate. More efficient elution of ribulose was achieved with 0.04 *M* borate.

The solutions containing free pentoses were first deionized by passing through a mixed bed of Dowex-50 and IR-4B resin at a rate of 3 ml. per minute. All the pentoses were recovered in the first 300 ml. This was reduced to dryness under reduced pressure, and the residue was dissolved in 25 ml. of 0.01 *M* sodium borate and passed down a Dowex-1 (borate) column. When necessary, the appropriate fractions were passed down Dowex-50 (H+ form) to convert the borate to the free acid and reduced to

dryness *in vacuo*. The residue was dissolved in methanol and the methyl borate removed by distillation. This process was repeated three times.

Preparation of Homogenate

Homogenates were prepared by grinding the required number of flies in an ice-cold Potter–Elvehjem type homogenizer consisting of teflon pestle and glass reservoir. The homogenate was filtered through muslin to remove grosser pieces of cuticle, and kept in crushed ice until used.

Results

G-6-P and 6-PG Dehydrogenases

The properties of G-6-P and 6-PG dehydrogenases in houseflies have been studied in detail (11).

Because of the stability of the pyranose ring of G-6-P in a neutral medium, the pyranose ring should remain intact during dehydrogenation with the consequent formation of the lactone of 6-phosphogluconic acid. The formation of this compound was studied by trapping it with hydroxylamine and thereby converting the lactone to the corresponding hydroxamic acid. The data in Table I indicate that formation of the hydroxamic acid depended upon the presence of G-6-P and TPN. In the absence of either substrate or coenzyme, no hydroxamic acid was formed.

TABLE I

DETECTION OF 6-PHOSPHOGLUCONOLACTONE AS A PRODUCT OF G-6-P OXIDATION

Expt. No.	G-6-P, moles/liter	Hydroxylamine, moles/liter	TPN, moles/liter	Phosphogluconhydroxamic acid formed, μM./g. tissue/2 hr.
1	2.6×10^{-3}	6.5×10^{-3}	1.5×10^{-4}	2.7
2	2.6×10^{-3}	1.84×10^{-3}	1.5×10^{-4}	3.3
3	2.6×10^{-3}	1.84×10^{-3}	1.5×10^{-4}	3.3
4	0	2.22×10^{-2} M	1.5×10^{-4}	0
5	2.2×10^{-3}	1.24×10^{-2} M	0	0

NOTE: The reaction mixture contained, in addition to those listed in the table, 0.016 *M* phosphate buffer, pH 6.5, and 3 ml. of homogenate. The total volume was 18.6 ml. The brei was prepared by homogenizing 100 2-day-old flies in 10 ml. of ice-cold 0.9% KCl for 1 minute. The incubation was carried out at room temperature. Two aliquots of 2 ml. were withdrawn for zero time before the reaction was started with TPN. All analyses were carried out in duplicate on 2 ml. of the incubation mixture.

Gluconolactonase Activity

The formation of 6-phosphogluconolactone as a product of G-6-P oxidation suggests the presence of an enzyme for the cleavage of the lactone as a preliminary step to the oxidative decarboxylation of the open chain compound, 6-PG. It is well known that the lactone may be hydrolyzed non-enzymatically at neutral or alkaline pH, but this reaction is rather slow and hence of doubtful physiological significance. The possible occurrence of an appropriate enzyme was therefore investigated.

TABLE II

GLUCONOLACTONASE ACTIVITY

Expt. No.	Lactone hydrolyzed, μM./g. tissue/30 min.	
	Housefly homogenate	Rabbit liver homogenate
1	39.6	212.4
2	43.5	—

NOTE: The reaction mixture contained 0.01 M. $MgCl_2$; 0.01 M. gluconolactone in 0.05 *M* phosphate buffer, pH 6.0, and 1 ml. of homogenate. Total volume was 5.0 ml. The homogenate was prepared by grinding 100 2-day-old female flies in 10 ml. of 0.9% KCl. The rabbit liver homogenate was prepared using 2.2 g. of fresh liver in 20 ml. of 0.9% KCl. All values were corrected for non-enzymatic hydrolysis by replacing the homogenate with an equal volume of boiled homogenate. The mixtures were incubated for 30 minutes at room temperature and analyzed for gluconolactone on 0.5 ml. samples in triplicate. The color was read at 540 mμ after 20 minutes.

The data in Table II indicate the presence in housefly homogenates of an active lactonase. The enzyme was also present in rabbit liver. The rate of hydrolysis of the lactone was linear with respect to enzyme concentration as shown in Fig. 1.

The enzyme was partially purified following the procedure of Brodie and Lipmann (7). Twenty-six grams of houseflies were blended in 150 ml. of distilled water for 1 minute at 1° C. After filtration through several layers of muslin, the extract was centrifuged at 20,000 × *g* for 15 minutes in a Servall angle centrifuge. The material was then heated for 10 minutes at 55° C. and cooled immediately. The coagulum was centrifuged for 10 minutes at 13,000 × *g* in a Servall angle centrifuge. The clear supernatant fluid was then fractionated with solid ammonium sulphate at 0.4 saturation.

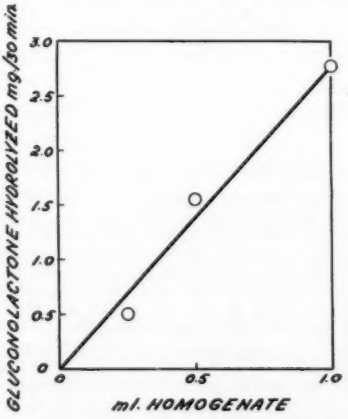


FIG. 1. For details see text to Table II.

After 1 hour, the precipitate was collected by centrifugation, dissolved in distilled water, and dialyzed overnight against 0.1 *M* phosphate buffer pH 6.0. The specific activity of the precipitate was about one-third that of the supernatant. By this simple procedure a fourfold purification was obtained with a recovery of 36%.

Enzymatic Conversion of 6-PG

(*i*) *Reaction Products and Stoichiometry*

The stoichiometry and the nature of the reaction products of the oxidation of 6-PG was studied by coupling this oxidation to the reduction of pyruvate in the presence of lactic dehydrogenase. The use of stoichiometric amounts of TPN is therefore avoided. The reduced TPN interacts with pyruvate and becomes reoxidized while pyruvate is in turn reduced. The reoxidized TPN can then react again with another molecule of 6-PG. This sequence of events thus continues until the substrates are utilized. Mehler *et al.* (39) have already shown that lactic dehydrogenase can react with TPNH.

The data shown in Table III indicate that the disappearance of 6-PG coincides with an appearance of equivalent amounts of CO_2, TPNH, and pentose as measured by the orcinol test. Further experiments showed this

TABLE III

STOICHIOMETRY OF PHOSPHOGLUCONATE OXIDATION

Expt. No.	Duration, minutes		Phosphogluconate, μM.	TPNH, μM.	CO_2, μM.	Pentose, μM.	Ketopentose, μM.
1	40	Initial	3.0		1.1	3.3	
		Final	1.2		2.8	4.7	
			−1.8		+1.7	+1.4	
2	40	Initial	9.0		1.7	2.9	
		Final	7.1		3.6	4.6	
			−1.9		+1.9	+1.7	
3	19	Initial		0			0
		Final		0.42			0.36
				+0.42			+0.36

NOTE: *Warburg study.*—The test system contained, in addition to the initial quantities shown in the table, 0.12 *M* glycylglycine buffer, pH 7.6; 7.4×10^{-3} *M* sodium pyruvate, 2.7×10^{-4} *M* TPN; and 0.5 ml. homogenate in a volume of 2.7 ml. Air was displaced by He; temperature was 25.2° C. The reaction was started by tipping TPN from one side arm and stopped by tipping 0.15 ml. of 90% TCA from the second side arm. Phosphogluconate was determined by the reduction of TPN with purified phosphogluconic dehydrogenase (23). The homogenate was prepared using 50 flies per 10 ml. of 0.9% KCl. In the control flasks, the substrate was replaced by an equal amount of buffer.

Spectrophotometric study.—The test system contained 8.3×10^{-2} *M* glycylglycine buffer, pH 7.5; 6.6×10^{-3} *M* $MgCl_2$; 1.6×10^{-4} *M* TPN; 1.33×10^{-3} *M* 6-PG; and 0.2 ml. enzyme (2.0 mg. protein). Total volume 3.0 ml. The reaction was started with TPN. After equilibrium was achieved, 0.2 ml. was withdrawn and added directly to the sulphuric acid used in the ketopentosephosphate determination (8). The concentration of TPNH was calculated from the extinction coefficient of 6.22×10^6 sq. cm./mole (22).

pentose to be primarily ketopentose, which absorbs at 540 mμ in the cysteine–carbazole reaction (18). The equation for the TPN-coupled oxidation of 6-PG by pyruvate in the presence of 6-PG dehydrogenase can therefore be written as follows:

$$\text{6-PG} + \text{TPN}^+ \longrightarrow \text{ketopentose phosphate} + \text{CO}_2 + \text{TPNH}.$$
$$\text{Pyruvate} + \text{TPNH} \longrightarrow \text{lactate} + \text{TPN}^+.$$

(*ii*) *Purification of 6-PG Dehydrogenase*

Early attempts to isolate the oxidation products failed because of the mixture of products which resulted from crude homogenates after prolonged incubation. Furthermore, it soon became apparent that adequate amounts of pentoses could not be obtained because of the interference by other enzymes, presumably transketolase and transaldolase. The enzyme was therefore purified to allow only a conversion of 6-PG to pentose phosphate. As will be shown later, this preparation is contaminated by phosphopentose isomerase and phosphoketopentose epimerase but no further conversion of the pentoses to either heptoses or hexoses occurred.

In a typical purification procedure, 96 g. of 2- to 4-day-old flies were blended in 250 ml. of cold distilled water for 2 minutes. After the mixture was centrifuged at 20,000 $\times$ *g* for 30 minutes an equal volume of saturated ammonium sulphate at 1° C. was added to the clear supernatant fluid. The precipitate was removed by centrifugation at 12,000 $\times$ *g* and discarded. The supernatant was saturated with ammonium sulphate. After 1 hour at room temperature the precipitate was collected by centrifugation at 25,000 $\times$ *g* for $\frac{1}{2}$ hour. The precipitate, dissolved in 42 ml. distilled water, was heated at 60° C. for 10 minutes and cooled immediately, and the coagulum was removed by centrifugation. To the supernatant fluid was added 3 ml. containing 100 mg. of protamine sulphate but no visible precipitate formed. Solid ammonium sulphate was added to give 50% saturation. The precipitate was removed by centrifugation at 10,500 $\times$ *g* for 15 minutes and dissolved in 16 ml. water. Because this preparation gave a cysteine–carbazole test, it was reprecipitated twice with solid ammonium sulphate at full saturation. The final solution, made up of the precipitate dissolved in 0.25 *M* glycylglycine buffer pH 7.5, gave no color with cysteine–carbazole. A summary of the purification procedure is presented in Table IV.

It is clear that approximately sixfold purification was achieved with approximately a 60% recovery. No attempt was made at further purification because these procedures were sufficient to eliminate transketolase.

The enzyme was relatively stable; after 5 days and 6 weeks at −10° C. it retained 90% and 80%, respectively, of its original activity.

(*iii*) *Accumulation and Identification of the Pentose Phosphates*

The accumulation of pentose phosphates was carried out by coupling the TPN oxidation of 6-PG with the reduction of pyruvate by lactic dehydrogenase as described in the above section. A typical reaction mixture consisted of 4.0×10^{-2} *M* glycylglycine buffer, pH 7.5; 6.25×10^{-2} *M* $MgCl_2$; 2.4×10^{-3}

TABLE IV

SUMMARY OF PURIFICATION OF 6-PG DEHYDROGENASE

Fraction No.	Volume, ml.	Units*/ml.	Total units	Protein, mg./ml.	Specific† activity	Recovery
Centrifuged extract	226	19.3	4362	14.7	1.3	—
$(NH_4)_2SO_4$ 0.5–1.0	42	115.8	4893	24.6	4.3	100
Heated supernatant fluid	35	101.4	3549	11.6	8.7	81
$(NH_4)_2SO_4$ 0–0.5 saturation	16	164.4	2630	22.3	7.4	60

* A unit of enzyme is defined as the amount of enzyme that reduces 1.0 mμM. TPN per minute.
† Specific activity is expressed as units per mg. of protein.

M 6-PG; 8.0×10^{-3} *M* sodium pyruvate; 0.2 ml. lactic dehydrogenase (1.52 mg. protein); 5.0 ml. of enzyme (50.5 mg. protein); and 3 mg. TPN. Total volume was 18.9 ml. The incubation was carried out in air at room temperature with periodic withdrawals of samples for analysis of ketopentose. Under the conditions of this large scale run all the 6-PG disappeared in 7 hours. The reaction was stopped by boiling for 5 minutes and brought to pH 5.2, and the sugar phosphates were dephosphorylated by the addition of 10 ml. of 0.2 *M* acetate buffer, pH 5.2, 4 ml. of 0.1 *M* $MgCl_2$, and 25 mg. acid phosphatase. The rate of dephosphorylation at 35° C. was determined via the inorganic phosphate released. At the completion of dephosphorylation, the reaction was stopped by boiling for 5 minutes. A check of the ketopentose before and after dephosphorylation revealed no change in concentration, suggesting no conversion of the ketopentose phosphates to aldopentose phosphates by the acid phosphatase. This dephosphorylated mixture was then passed through a Dowex-1 borate column as already described.

The data in Fig. 2 indicate that the two major ketopentoses (peaks II and III) corresponded to D-xylulose and D-ribulose. Orcinol tests on aliquots of fractions 22 to 27 showed a double banded spectrum with peaks at 540 mμ

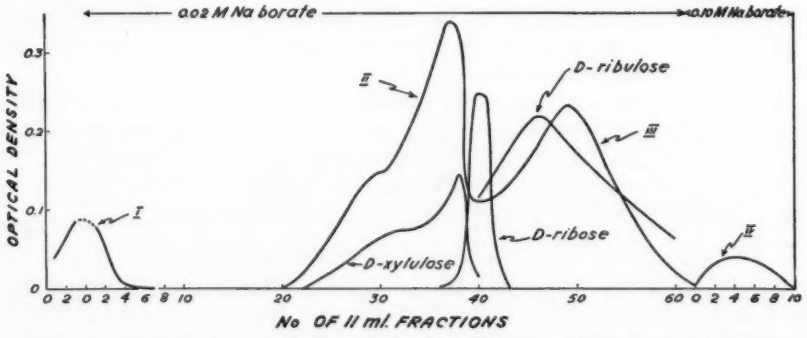


FIG. 2. Column chromatography of products of 6-PG oxidation. For details of experiment see text.

and 670 mμ. The ratio of the two peaks was 0.52, in good agreement with the known ratio for D-xylulose (21, 48). For the subsequent fractions this ratio dropped to an average value of 0.27 suggesting the presence of an interfering substance, most probably D-ribose. As seen in Fig. 3, the orcinol test on an aliquot of tube 49 showed a characteristic two-banded spectrum of D-ribulose with a value of 0.88 for the ratio of the two peaks. A further analysis of tubes 42 to 58 also showed the presence of ribulose with an average ratio for the two peaks of 0.89. This is in good agreement with published values (21, 48). Furthermore, the components of tubes 42–58 reacted completely within 15 minutes in the cysteine–carbazole test; the components in tubes 22 to 40 required about 3 hours to reach maximum color intensity; reactions which indicate D-ribulose and D-xylulose respectively (53).

An analysis of the free pentoses in tubes 28 to 40 was also carried out by paper chromatography. The data in Table V indicate the presence of two components with R_f's in both solvents identical with those of D-xylulose and D-ribose. The color reactions after spraying of the chromatograms were also identical with the authentic samples.

TABLE V

PAPER CHROMATOGRAPHY OF PEAK II

Solvent	Reaction products, R_f	Xylulose, R_f	Ribose, R_f
77% acetone	0.72, 0.66	0.72	0.66
	0.80	0.80	—
	0.77	0.78	—
Acetone – water – boric acid	0.56, 0.88	0.57	0.88
	0.61, 0.76	0.61	0.76
	0.53, 0.82	0.53	0.82

NOTE: The contents of tubes 28 to 40 were passed down Dowex-50 (H^+ form). The effluent was reduced to dryness *in vacuo* and the boric acid removed as methyl borate with methanol. The slight residue was taken up in 0.3 ml. and aliquots were chromatographed at room temperature overnight. The sugars were visualized by spraying appropriate reagents as described in the text.

A further confirmation of the identity of the components in this fraction was obtained by eluting both the xylulose and ribose and subjecting them to the orcinol test. Difficulty was encountered, however, because of the presence of elutable substances in the paper which reacted positively in the orcinol test. This was overcome by prewashing the paper in the chromatographic solvents and then eliminating the unwashed paper along a line a few inches below the level of the solvent front. Correction was made for a blank of equivalent size to the test spot.

The data in Fig. 3 indicate that the component with the $R_f = 0.88$ exhibits only one strong absorption maximum at 670 mμ. The ratio of the optical densities at 540 and 670 mμ was 0.21. The other component ($R_f = 0.56$) exhibits an absorption spectrum with peaks at 670 mμ and 540 mμ.

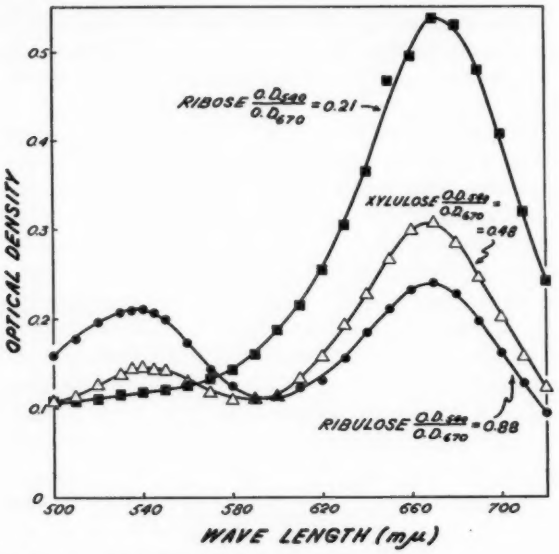


FIG. 3. Absorption spectra of the oxidation products of 6-PG.
●——● Orcinol test on an aliquot of tube 49.
△——△ Orcinol test on the eluate of the component with $R_f = 0.56$ after the contents of tubes 28–40 were chromatographed in acetone–water–boric acid.
■——■ Orcinol test on the eluate of the component with $R_f = 0.88$ after the contents of tubes 28–40 were chromatographed in acetone–water–boric acid.

The ratio of these peaks was 0.48. Both of these ratios are in good agreement with that for D-ribose and D-xylulose (21, 48). It is evident, therefore, that the products from the oxidation of 6-PG are D-ribulose, D-xylulose, and D-ribose. This, in turn, suggests that our preparation of 6-PG dehydrogenase is contaminated with a phosphopentose isomerase and phosphoketopentose epimerase.

Phosphopentose Isomerase

The occurrence of D-ribulose and D-ribose as products of 6-PG oxidation indicates the presence of phosphopentose isomerase. A direct assay for this enzyme was made by incubating 80 μM. of R-5-P with 1 ml. of enzyme (10.1 mg. protein) in a total volume of 5 ml. After 2½ hours, the isomerization was completed as measured by the ketopentose formation. The reaction was stopped by boiling for 5 minutes and then adjusted to pH 4.0. The pentose phosphates were dephosphorylated at 37° C. in a medium containing 1 ml. of 0.2 *M* acetate buffer, pH 4.0; 0.3 ml. of 0.1 *M* cysteine, pH 4.0, 0.7 ml. of 0.1 *M* $MgCl_2$, and 4 ml. of acid phosphatase as prepared according to Hochster (21). The dephosphorylation was completed in 1½ hours, and the reaction stopped by boiling. The mixture was deionized as already described and then chromatographed on a Dowex-1 (borate) resin.

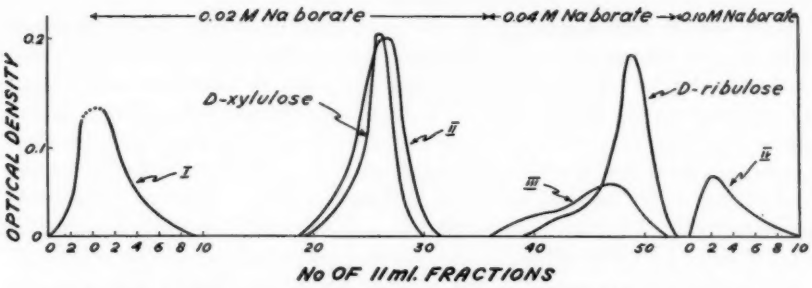


FIG. 4. Column chromatography of the products of isomerized R-5-P. For details see text.

The data in Fig. 4 indicate the presence of two ketopentoses corresponding to authentic samples of D-xylulose and D-ribulose. Maximum color density in cysteine–carbazole was achieved in 15 minutes with peak III whereas 2–3 hours were required with peak II. As already shown, these properties are characteristic of D-ribulose and D-xylulose.

The data in Figs. 2 and 4 indicate the presence of two other substances which gave a pinkish-blue color in the cysteine–carbazole test. Peak I passed rapidly through the column in 0.01 *M* borate and 0.02 *M* borate as if it did not form a borate complex. The absorption spectrum of this substance in orcinol showed two peaks, one at 540 mμ, and the other at 670 mμ. The ratio of the two peaks was 0.52. The rate of color development in cysteine–carbazole was reminiscent of xylulose in that 2 to 3 hours were required for maximum color development. The absorption spectrum of this substance in cysteine–carbazole showed a typical ketopentose peak at 540 mμ. The indications are, therefore, that this substance is L-xylulose but a definite identification must await further work.

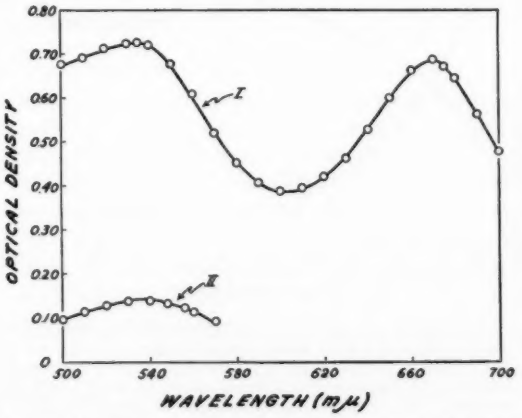


FIG. 5. Color reactions of 3-ketopentose in tube 4 of peak IV (Fig. 4). Curve I, orcinol reaction. Curve II, cysteine–carbazole reaction.

The fourth peak appearing in the incubation mixtures was eluted with 0.10 *M* sodium borate. This substance gave a positive cysteine–carbazole and orcinol test. As seen in Fig. 5, the maximum in the cysteine–carbazole spectrum occurred at 540 mμ. The double-banded orcinol spectrum of this substance, with maxima at 540 mμ and 670 mμ and a ratio of 1.06 for these peaks suggested that it might be a 3-ketopentose as already found by Ashwell and Hickman (2) and Dickens and Williamson (13). None of these peaks appeared when R-5-P was incubated only with the acid phosphatase indicating that these substances were not the result of phosphatase action on R-5-P.

The rate of isomerization of the aldopentose to the ketopentose is seen in Fig. 6. The data show that for every mole of aldopentose which disappeared 1 mole of ketopentose appeared. The ketopentoses were treated with an equal volume of 1.5% bromine water in the presence of 500 mg. barium carbonate for 30 minutes. After removal of the bromine by aeration, the solution was treated with cysteine–carbazole. No change in the concentration of ketopentoses was observed after this oxidation.

The data in Fig. 7 indicate that the amount of xylulose in proportion to the total ketopentose level increased with time of incubation suggesting that ribulose was being converted to xylulose. If the difference in optical density between 120 minutes and 10 minutes is used as an approximate measure of xylulose, then very little, if any, xylulose formation occurred within ½ minute incubation. Some xylulose appeared after 2 minutes and thereafter the level increased with increasing time of incubation.

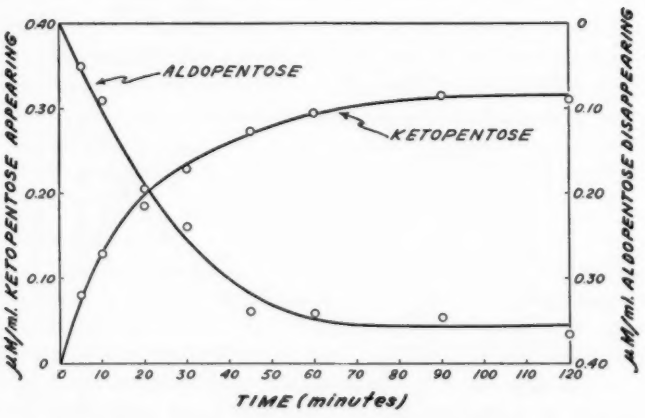


FIG. 6. Rate of disappearance of R-5-P and appearance of ketopentose phosphates. The reaction mixture contained 0.20 *M* glycylglycine buffer, pH 7.5; 2.0×10^{-3} *M* R-5-P (pH 7.5) and 1.5 ml. of the enzyme (0.30 mg. protein). The ketopentose analyses were carried out in duplicate on aliquots which were added to 6 ml. of H_2SO_4 used on the cysteine–carbazole test. The orcinol tests were carried out on aliquots added to 10% TCA to stop the reaction at various periods of time. The aldopentose values were corrected for interference by ketopentoses using an average extinction coefficient of 18,000 (28, 53) for the mixture of Ru-5-P and Xu-5-P.

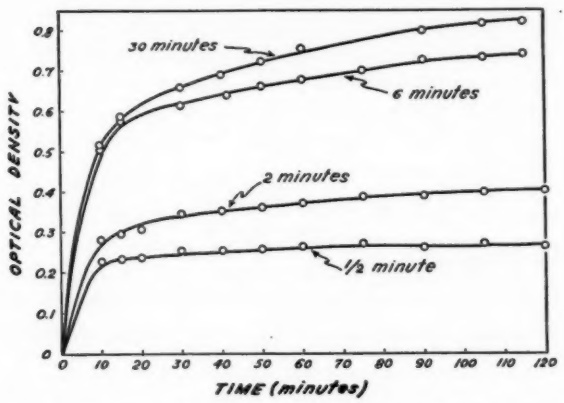


FIG. 7. The formation of xylulose phosphate as a function of time. The reaction mixture contained 0.21 *M* glycylglycine buffer, pH 7.5; 1.5×10^{-3} *M* R-5-P, pH 7.5 and 1.5 ml. enzyme (6-PG dehydrogenase preparation 3.78 mg. protein). At various periods of time 2 ml. aliquots were withdrawn and added to 0.2 ml. of 11.6 *N* HCl to stop the reaction. The pH was adjusted to 5.3 and each aliquot dephosphorylated with 20 mg. acid phosphatase at 37° C. The rate of color development was determined on 1 ml. aliquots using the cysteine–carbazole test at 540 mμ. All values were corrected for the isomerase activity of the acid phosphatase.

The data presented in this section may therefore be interpreted by the following sequence of events:

D-R-5-P ⟷ D-Ru-5-P ⟷ 3-ketopentose phosphate ⟷ D-Xu-5-P

It has already been shown that this series of reactions is reversible (13, 28, 54). The conversion of D-Ru-5-P to D-Xu-5-P is probably catalyzed by phosphoketopentose epimerase.

Phosphoketopentose Epimerase

The gradual appearance of xylulose in a preparation with Ru-5-P or R-5-P as substrates as shown in the previous section suggests the presence of an enzyme which catalyzes the interconversion of ribulose to xylulose. A direct demonstration of this enzyme was obtained by incubating Ru-5-P with the 6-PG dehydrogenase preparation. This permitted the accumulation of Xu-5-P. As shown in Fig. 8, no Xu-5-P appeared when Ru-5-P was incubated for 60 minutes with a boiled preparation. However, an active preparation permitted the accumulation of xylulose as shown by the time necessary to achieve maximum color development in cysteine–carbazole.

Conversion of Pentose Phosphate to Hexose Monophosphate

(*i*) *Accumulation and Identity of Products*

When R-5-P was incubated for any period of time with housefly extracts, it became evident that more ribose phosphate disappeared than could be

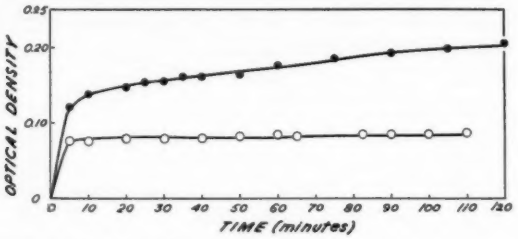


FIG. 8. Phosphoketopentose epimerase.
The reaction mixture contained 1.1×10^{-3} *M* Ru-5-P; 0.1 ml. of enzyme (6-PG dehydrogenase preparation, 1.5 mg. protein) and 0.013 *M* Tris buffer (pH 7.5) in a total volume of 0.8 ml.
The control consisted of boiled enzyme. After 1 hour incubation, the reaction was stopped by boiling for 2 minutes. The pH of the mixture was reduced to 5.0 and the pentose phosphates dephosphorylated in the following medium: 0.5 ml. of 0.5 *M* sodium acetate (pH 5.0); 0.05 ml. of 0.1 *M* $MgCl_2$; 0.05 ml. of 0.1 *M* cysteine (pH 7.0); and 2 mg. acid phosphatase. After a 3-hour incubation at 35° C., the reaction was stopped by boiling in a water bath for 2 minutes. The solution was reduced to dryness *in vacuo* and dissolved in 0.2 ml. distilled water. Aliquots were reacted in the cysteine–carbazole test performed as described in text.
○———○ Boiled control. ●———● Test preparation.

accounted for by its isomerization to ribulose, especially since the data of Horecker *et al.* (28) indicate a close similarity in the chromogenic values of both these pentoses in the orcinol reaction. Tests were therefore made for other potential intermediates which have already been implicated in plant and mammalian metabolism (3, 24, 27). Housefly flight muscle has been shown to possess an active transketolase and transaldolase (10) while heptoses and trioses were also found in the metabolism of pea aphids (41).

The data in Table VI indicate that heptulose, hexose, and triose phosphates were all products of the reactions involving the disappearance of R-5-P. Furthermore, the amounts of intermediates seem to depend upon the length of incubation.

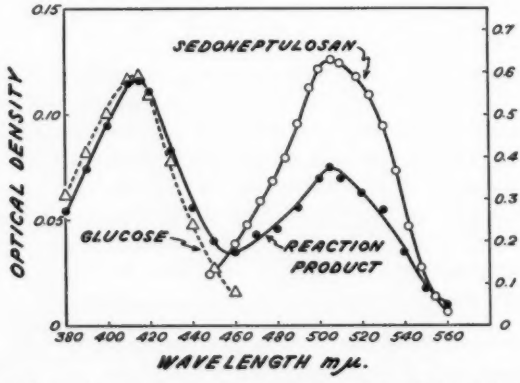


FIG. 9. The absorption spectrum of the products of R-5-P breakdown reacted with sulphuric acid – cysteine as well as sedoheptulose and glucose. For details see Table VI. The curve was corrected for endogenous absorption. Incubation time was 210 minutes.

The heptulose was identified as sedoheptulose after reaction with sulphuric acid–cysteine. The typical absorption spectrum of the reaction products is seen in Fig. 9. The reaction products exhibit two peaks; one around 505 mμ which is identical with that of sedoheptulose and the other at 415 mμ which corresponds to a hexose.

(*ii*) *Stoichiometry of R-5-P Breakdown*

The data in Table VI suggest that after 30 minutes, approximately 2 moles of pentose phosphate are utilized for every mole of heptulose phosphate that is formed, though only 83% of the theoretical conversion was obtained. After 60 minutes' incubation, however, the stoichiometry of 1 mole of heptulose and hexose phosphate arising from 2 moles of pentose phosphate was fully achieved.

TABLE VI

DEGRADATION OF R-5-P BY HOUSEFLY TRANSKETOLASE

	30 minutes	60 minutes
Pentose consumed	10.8	16.1
Heptulose formed	4.3	7.1
Hexose formed	0.2	0.8
Triose formed	1.3	0.8

NOTE: The reaction tube contained 8×10^{-4} *M* R-5-P, pH 7.5; 0.05 *M* glycylglycine buffer, pH 7.6; 5.8×10^{-5} *M* ThPP; 2.7×10^{-2} *M* NaF; 3.0 ml. tissue and enough water to total volume of 30 ml. The extract was prepared from 50 female flies which had been starved from 1–2 days. The homogenate was prepared in distilled water and centrifuged at 10,000 × *g* for 10 minutes at 1° C. Aliquots were withdrawn, deproteinized with TCA in all instances except that for triose measurements where the deproteinization was performed by boiling for 3 minutes. The temperature was 23° C. In the control incubation the active enzyme was replaced by boiled enzyme. The values for alkali-labile phosphorus at the various incubation periods were corrected for changes in inorganic phosphate.

This apparent lag, previously noticed by Axelrod *et al.* (3) and Kinoshita *et al.* (32) and also evidenced in the data of Horecker *et al.* (27), was studied further. The data in Table VII indicate that the achievement of the theoretical stoichiometry between pentose breakdown and heptulose formation depends upon the length of the incubation period and upon the substrate used. With R-5-P as substrate full stoichiometry was achieved after 45 minutes. The addition of Ru-5-P improved the situation considerably. It was apparent, therefore, that the lag is not entirely due to a transformation of R-5-P to Ru-5-P. The rate-limiting step appears to be the formation of another compound in the sequence of events involving the transformation of Ru-5-P to heptulose-7-P.

It has been suggested that such a conversion proceeds via a 2-carbon fragment "active glycolaldehyde." De la Haba *et al.* (19) have shown that transketolase prepared from yeast possesses the ability to decarboxylate

TABLE VII

STOICHIOMETRY OF PENTOSE PHOSPHATE CONVERSION TO SEDOHEPTULOSE PHOSPHATE

	Time, minutes					
	10	20	30	45	60	90
Pentose added	% of theoretical = $\frac{\text{heptulose}}{\frac{1}{2}\text{ pentose}} \times 100$					
R-5-P*	47	70	87	100	100	74
R-5-P + Ru-5-P†	59	88	100	100	100	100
R-5-P + Ru-5-P + hydroxypyruvate‡	100	100	100	100	100	100

NOTE: For composition of the reaction mixture see Table VI.
* 8×10^{-4} *M* R-5-P.
† 8×10^{-4} *M* R-5-P + 4.3×10^{-4} *M* Ru-5-P.
‡ 8×10^{-4} *M* R-5-P + 4.3×10^{-4} *M* Ru-5-P + 1.4×10^{-2} *M* hydroxypyruvate.
The extinction coefficient of Ru-5-P was taken as 20,000 (28). All pentose values were corrected for interference by heptulose.

hydroxypyruvate presumably to glycolaldehyde, which, in the presence of an appropriate aldehyde acceptor, forms ketopentose phosphate. Furthermore, incubation of hydroxypyruvate and R-5-P with transketolase resulted in the formation of heptulose phosphate. It was reasoned, therefore, that if the transfer of "glycolaldehyde" is the rate-limiting step, then the lag should be eliminated by the addition of hydroxypyruvate. That such is the case is seen in Table VII. The theoretical stoichiometry of 2-pentose phosphates disappearing for every mole of heptulose phosphate appearing is achieved immediately.

(iii) Time-course Formation of Products of R-5-P Breakdown

The data in Table VI indicate that hexose and triose are other intermediates in the breakdown of R-5-P. Furthermore, the level of hexose phosphates appears to be a function of time. More information was therefore sought on the time interrelationships of these intermediates.

The data in Fig. 10 represent a typical sequence of events. The utilization of R-5-P is very rapid. Approximately 50% of the pentose disappeared in 20 minutes. After 2 hours, approximately 72% of the pentose was consumed. Longer periods of incubation indicated the attainment of an equilibrium. The participation of sedoheptulose and triose as intermediates in the conversion of R-5-P is indicated by the order in which the products appear. Triose and sedoheptulose accumulate very quickly with triose appearing perhaps even sooner than sedoheptulose. This early appearance of triose could be explained by assuming that the initial degradation of ribulose phosphate occurs via a 2-carbon fragment. The rate of appearance of sedoheptulose would depend on the rate of combination of the 2-carbon fragment with an appropriate acceptor. After 60 minutes, the level of sedoheptulose decreases, while the amount of hexose monophosphate increases. Very little, if any, hexose monophosphate was formed during the early stages of incubation.

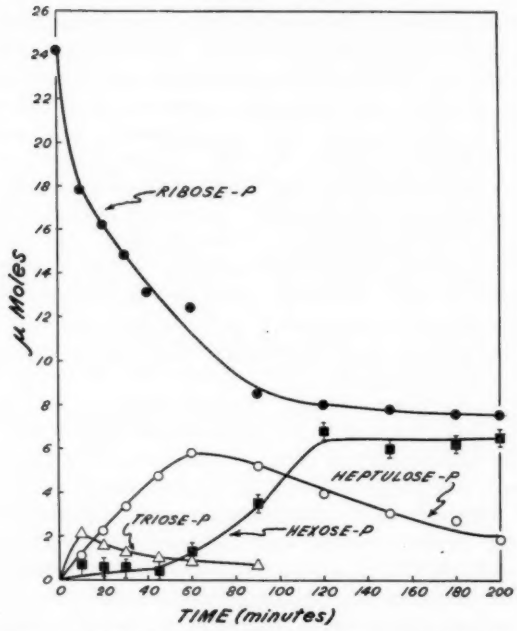
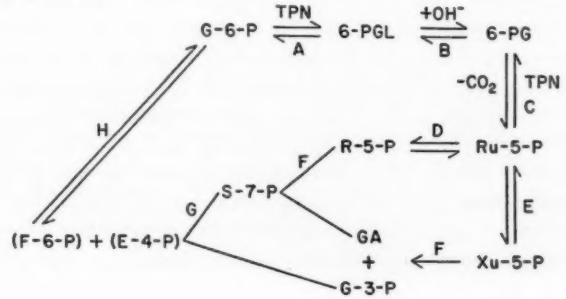


FIG. 10. Time course of R-5-P utilization. For the composition and treatment of the reaction mixture see Table VI.

Horecker and Smyrniotis (24) have described some of the properties of transaldolase, which is involved in the conversion of heptulose-P to hexose-P. Furthermore, it has been shown that an intermediate in this conversion was erythrose-P (24, 34); Ballou *et al.* (4) have shown erythrose-P to be readily susceptible to alkaline hydrolysis. But all attempts to demonstrate by alkaline hydrolysis an accumulation of erythrose-P in our preparations during the conversion of heptulose-P to hexose-P failed.

Discussion

The reactions discussed in this paper can be summarized in a cyclic mechanism as follows:



This cyclic process in the housefly as shown here is catalyzed by a team of enzymes: A, G-6-P dehydrogenase; B, 6-phosphogluconolactonase; C, 6-PG dehydrogenase; D, phosphopentose isomerase; E, phosphoketopentose epimerase; F, transketolase; G, transaldolase; and H, phosphohexose isomerase. Only the first two steps are oxidative. The second step also involves a decarboxylation yielding Ru-5-P. All the succeeding steps represent group transfer reactions some of which may presumably proceed anaerobically in insects, as in other organisms (6, 18). As a result of these transfer reactions, F-6-P is presumably produced which can re-enter this cycle after isomerization to G-6-P by phosphohexose isomerase, an enzyme already shown to be present in the housefly (9). By means of this cyclic mechanism G-6-P could be completely oxidized to CO_2 without the intervention of the Embden–Meyerhoff pathway or the Krebs citric acid cycle. The over-all complete oxidation can be written as follows:

$$\text{G-6-P} + 3O_2 \longrightarrow 6\ CO_2 + \text{inorganic phosphate.}$$

Although the reversibility of some of these reactions in the housefly has not been shown, there appears little doubt that they are reversible. Thus, Horecker and Smyrniotis (25) have demonstrated the reversibility of G-6-P oxidation as well as the reductive carboxylation of Ru-5-P to 6-PG by yeast preparations (26). The reactions catalyzed by transketolase and transaldolase are also reversible (24, 27). Furthermore, when R-5-P was incubated with housefly extracts (Fig. 10) an equilibrium appears to be attained when 72% of the pentose disappeared. Horecker *et al.* (25) and Glock (18) found that during the formation of sedoheptulose and hexose from pentose by liver preparations and spinach transketolase, approximately 75 to 80% of the pentose disappeared before equilibrium was established.

Pentose conversion to heptulose reaches the theoretical value of 2 moles of pentose per mole of sedoheptulose only after a lag period. Similar findings have been reported for plants by Axelrod *et al.* (3) and for corneal epithelium by Kinoshita *et al.* (32). It appears that the conversion of ribulose-5-P to heptulose phosphate by housefly preparations involves an intermediate reaction, most probably, the formation of a C_2 fragment. This assumption is strengthened by the finding that triose makes its initial appearance in our reaction much sooner and in greater amount than sedoheptulose (Fig. 10). It appears, therefore, that the rate-limiting step in this sequence of events is not the rate of production of the C_2 fragment but rather its rate of transference and combination with an appropriate aldehyde acceptor, presumably R-5-P. As our data show, the addition of hydroxypyruvate, a potential glycolaldehyde donor, eliminates the lag completely. Although previous workers have suggested a C_2 fragment, and de la Haba *et al.* (19) have shown the formation of sedoheptulose from hydroxypyruvate and R-5-P by highly purified transketolase, the present evidence appears to be the first for the participation of a C_2 intermediate in the sequence of events in crude extracts, thereby giving it greater physiological significance.

As for the formation of the hexose ester from sedoheptulose-P in insects, little can be said at present. The order of the appearance of the hexose ester suggests that it is probably formed from sedoheptulose. Other studies have shown that transaldolase catalyzed the reaction

$$\text{S-7-P} + \text{G-3-P} \rightleftharpoons \text{F-6-P} + \text{E-4-P}.$$

The present study makes no attempt at an identification of the hexose ester, although probably it is F-6-P. All attempts to demonstrate an accumulation of E-4-P in insect extracts by virtue of the alkali-labile P (4) failed. This is not surprising, however. Recent studies have shown that tetrose is a very reactive molecule and may re-enter the cycle (34) or participate in other reactions (52).

It now appears that xylulose-5-phosphate is a key intermediate in the pentose metabolism in both mammals and microorganisms (2, 13, 21, 49, 53). It is the product of catalysis of either xylose isomerase or phosphoketopentose epimerase. Stumpf and Horecker (53) have recently described some properties of this epimerase. The mechanism of the formation of Xu-5-P from Ru-5-P by the epimerase is as yet not clear, but Ashwell and Hickman (2) have postulated a 3-ketopentose intermediate. Probably the real significance of Xu-5-P was found by Srere *et al.* (51) when they showed it to be the substrate for transketolase. Our demonstration and identification of Xu-5-P for the first time as an intermediate in the metabolism in insects extends the universality and significance of this intermediate in carbohydrate metabolism.

Acknowledgments

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THE ACUTE ORAL TOXICITY OF SPIRAMYCIN[1]

ELDON M. BOYD

Abstract

Spiramycin was administered as a suspension by stomach tube in a range of doses to 46 young male albino rats, with 16 controls, and to 14 small mongrel bitches, with eight controls. The oral acute LD_{50} was found to be 9.4 ± 0.8 g. (mean ± S.D.) per kg. body weight in rats and 5.2 ± 1.6 g. per kg. body weight in dogs. From these values, the oral acute LD_{50} in man was estimated to be of the order of 1 to 2 g. per kg. body weight or 20 to 40 times the usual therapeutic dose. The clinical effects of these acutely toxic oral doses in rats and dogs were anorexia, vomiting (dogs only), diarrhea, and lassitude, progressing to prostration, pallor, a fall in body temperature, cessation of respiration, and death usually within 48 hours. At autopsy the stomach and intestines were distended with gas and liquid, the tunica propria and submucosa were acutely congested, and there was excessive necrosis and desquamation of the surface epithelium. Areas of acute necrosis were found in the hepatic cells and sinusoids of the liver, and in the (mostly distal) convoluted tubules of the kidney. The cause of death, therefore, was an acute fulminating gastroenteritis accompanied by acute regional necrosis of the liver and kidneys, produced by the orally administered lethal dose of spiramycin.

Spiramycin is the name approved by the British Pharmacopoeia Commission (2) for an antibiotic agent produced by *Streptomyces ambofaciens*, a species of fungus discovered in the soil of Northern France and found to produce *two* antibiotics, congocidin (5) and spiramycin (11). It became available in 1954 (12) under the name Rovamycin(e) or 5337 R.P. It is a pale, cream-colored, amorphous base, with a bitter taste, almost insoluble in water but capable of forming water-soluble salts such as the adipate and sulphate. Coated tablets each containing 250 mg. of the base are available for clinical use. The antibacterial spectrum *in vitro* is similar to that of erythromycin (11). The LD_{50} in mice has been reported by Pinnert-Sindico and her associates (11) to be over 5 g. per kg. orally, 1.5 to 2.0 g. per kg. subcutaneously, and 0.15 to 0.25 g. per kg. intravenously.

Oral doses of 2 g. in man produce a level of 0.3 mg. per 100 ml. of blood in 2 hours (8), lesser levels appearing in cerebrospinal fluid. Ninety per cent of the blood spiramycin disappears by 24 hours, and only 10% is collected unchanged in urine (8). Clinical reports (1, 3, 7, 8, 9, 12) indicate that spiramycin should be classified with penicillin and other antibiotics effective primarily against Gram-positive organisms. Spiramycin has been found active against some (1) but not all (10) penicillin-resistant organisms.

Pathological changes due to acutely lethal oral doses of spiramycin have not been described in the literature, apart from statements that the oral LD_{50} in mice is over 5 g. per kg. body weight (4, 7, 8, 11, 13). Gastralgia, nausea, vomiting, and diarrhea have been reported in some 1 to 3% of patients given clinical doses of spiramycin (8).

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Contribution from the Department of Pharmacology, Queen's University, Kingston, Ontario. A paper upon this work was presented before the meeting of the Pharmacological Society of Canada at the University of Alberta, Edmonton, Alberta, on June 18, 1957. This investigation was supported by a grant from Poulenc Limited of Montreal.

Methods

Spiramycin base was triturated to a very fine powder and converted into a 50% (w/v) suspension in distilled water with the aid of about 1% powdered tragacanth. The suspension was prepared just before oral administration to albino rats, by means of a syringe and point-dulled 3 in. needle, and to dogs using a large syringe and stomach tube.

Young male albino rats, 2 to 3 months old and weighing 200 to 350 g., were fasted 16 hours in individual rat metabolism cages. Then they were given spiramycin in oral doses of 0, 1, 2, 4, 6, 8, 9, and 10 g. per kg. body weight. Food (Purina Fox Chow checkers) and water consumption, body weight, stool and urine output, clinical symptoms, and colonic temperature were recorded every 24 hours or as indicated by the effects being obtained. If and when death appeared imminent, the animals were observed more closely so that an autopsy could be performed immediately after death. Of the 10 autopsies performed on spiramycin-treated rats, seven were done immediately after death and the remaining three within an estimated maximum of 4 to 5 hours.

At autopsy, wet weight and water content were measured upon the stomach, small bowel, caecum, colon, liver, adrenals, kidneys, testes, skeletal muscle (ventral abdominal wall), and residual carcass (reduced to an homogenate in a Waring blendor). The organs of the gastrointestinal tract were washed free of their contents and dried on blotting paper before being weighed. Blocks of representative organs were put into a formol fixative and microscopic sections prepared with a hematoxylin–phloxine–saffron stain.

Ten duplicate experiments were run on rats at the 1 g. per kg. dose level, two at each of the 2 and 4 g. per kg. dose levels and eight at each of the 6, 8, 9, and 10 g. per kg. dose levels. Sixteen rats were used as controls and received no spiramycin.

Twenty-two mongrel bitches weighing from 5 to 10 kg. were adequately fed and watered and were observed to maintain constant body weights for 2 weeks prior to the administration of spiramycin. Four of these dogs were given 1 g. per kg. body weight of spiramycin orally, three were given 3 g. per kg., two were given 4 g. per kg., three were given 6 g. per kg., and two were given 9 g. per kg. Eight dogs were maintained as controls and received no spiramycin. Within 15 to 30 minutes after the administration of the spiramycin all of the dogs vomited except one of those receiving the smallest dose. None of these dogs died and from the dry weights of the vomitus it was estimated that most of the spiramycin had been ejected. Intramuscular doses of 25 mg. per kg. of chlorpromazine hydrochloride failed to prevent the spiramycin-induced vomiting but intraperitoneal doses of 25 mg. per kg. of pentobarbital sodium, or sufficient to produce light surgical anaesthesia, did inhibit the vomiting reflex and enable the spiramycin to be retained. Those dogs receiving 6 and 9 g. per kg. body weight of spiramycin subsequently died.

Results

A. Albino Rats

The initial clinical effects of spiramycin in doses of 2 g. per kg. body weight and over were anorexia, diarrhea, and lassitude. Ten rats died, three within 24 hours, six between 24 and 48 hours, and one at the end of the seventh day after giving spiramycin. The symptoms during the 6 hours preceding death were pallor of the conjunctiva, ears, and feet, prostration, a slow respiratory rate, and a fall in the colonic temperature. The colonic temperature averaged $99.6 \pm 0.2°$ F. (mean $\pm$ S.D.) in the controls, $97.8 \pm 0.9°$ F. 24 hours after a nonlethal dose, and $92.6 \pm 0.6°$ F. just prior to death. The latter two means were significantly ($P < .01$) different from the control mean. One rat was observed to enter cardiac failure 2 minutes after respirations had stopped.

A correlation of percentage mortality versus dose of spiramycin is shown in Fig. 1 in which a regression line has been drawn, $\pm$ its standard deviation, from the estimating equation, $Ye = (16.4X - 105) \pm 12$. This equation was calculated by the method of least squares through solution of the two normal equations of Croxton (6). The oral LD_{50} for the albino rat, estimated by this technique, was found to be 9.4 ± 0.8 g. per kg. body weight.

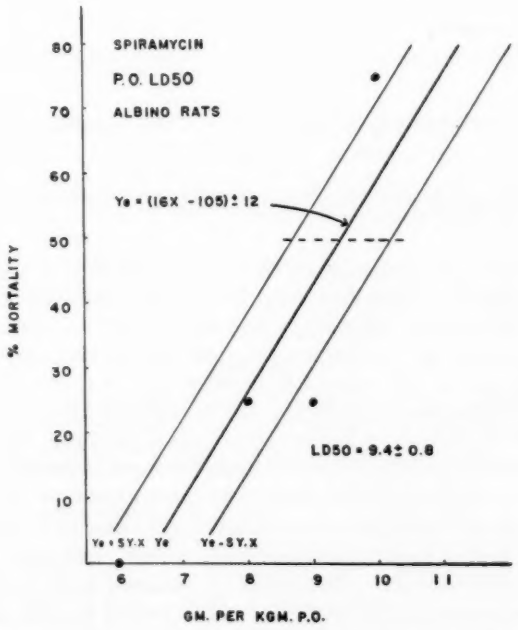


FIG. 1. A correlation of the percentage mortality versus oral dose of spiramycin administered to albino rats. The oral dose is expressed as g. per kilogram (kg.) body weight, given orally (P.O. = per os).

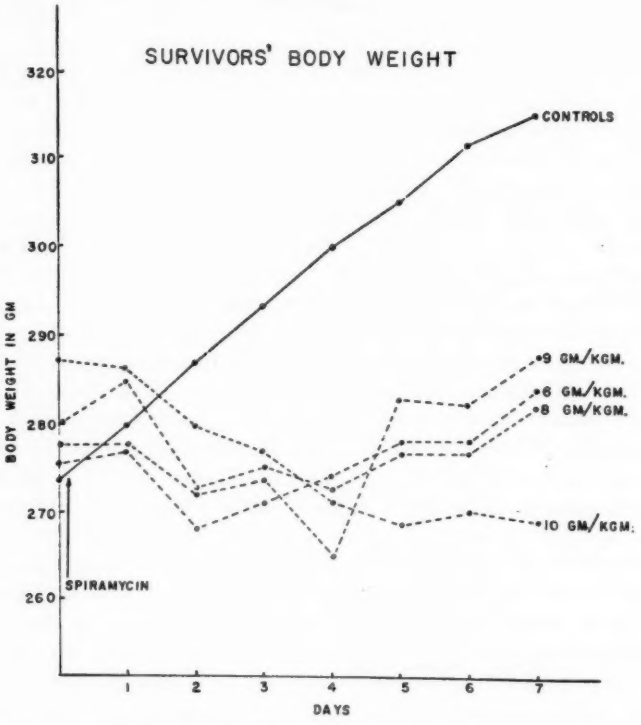


FIG. 2. Loss of body weight by albino rats surviving oral administration of doses of spiramycin within the lethal range.

Mean shifts in body weight of survivors receiving 6, 8, 9, and 10 g. per kg. are shown in Fig. 2. There was a significant ($P < .001$) loss of body weight in the days immediately following administration of spiramycin. Recovery of the normal rate of growth occurred toward the end of 1 week after the 6, 8, and 9 g. doses and in the second week after the 10 g. dose. Loss of body weight was associated with a reduction in the intake of food (Fig. 3) and water, a reduction in the output of formed stool, and a diarrhea. An autopsy performed on three rats which recovered revealed no macroscopic abnormalities and no shifts in weight or water content of the organs 4 weeks later.

The average loss of weight in 10 rats which died was 10.7 ± 6.1 % of body weight. The stomach and intestines were acutely inflamed and distended with a dark yellowish fluid. The stomach contained some unabsorbed spiramycin. There was considerable gas, partially carbon dioxide, in the stomach and occasionally in the caecum and colon. Weights of organs removed at autopsy were expressed as a percentage of body weight. The only significant changes found following spiramycin were losses of wet weight in the small

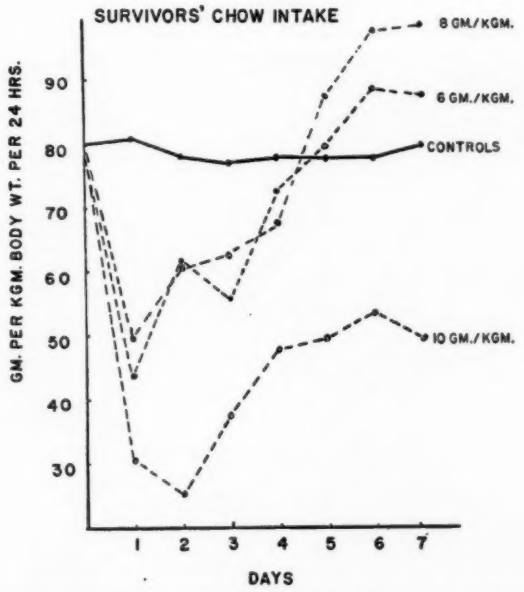


FIG. 3. Reduction of food intake by albino rats surviving oral administration of doses of spiramycin within the lethal range.

bowel, caecum, and colon (Table I). As noted in Table II, there was a significant ($P < .05$) loss of water by carcass and by most organs analyzed except liver, in which water levels were augmented. These results indicate that lethal doses of spiramycin produced an acute gastroenteritis with diarrhea and loss of body water.

Microscopically, sections of the gastrointestinal tract exhibited marked dilatation of the capillaries of the tunica propria and of blood vessels in the submucosa (Fig. 4A). Desquamation of the surface epithelium was in excess

TABLE I

THE WET WEIGHT OF TISSUES REMOVED AT AUTOPSY FROM ALBINO RATS GIVEN AN ACUTELY LETHAL DOSE OF SPIRAMYCIN ORALLY

(Wet weight is expressed as percentage of body weight)

Tissue	Spiramycin-treated $\bar{X}s \pm \sigma$	Controls $\bar{X}c \pm \sigma$	$\frac{\bar{X}s - \bar{X}c}{\bar{X}c} \times 100$	P (difference equals zero)
Small bowel	2.078 ± 0.440	2.894 ± 0.588	−28	.001
Colon	0.517 ± 0.095	0.675 ± 0.070	−23	.001
Caecum	0.379 ± 0.071	0.452 ± 0.086	−16	.025
Stomach	0.510 ± 0.072	0.502 ± 0.052	+ 2	.7
Liver	4.39 ± 0.49	4.38 ± 1.01	0	1.0
Adrenals	0.0151 ± 0.0098	0.0138 ± 0.0040	+ 9	.9
Kidneys	0.793 ± 0.077	0.818 ± 0.088	− 3	.5
Testicles	1.11 ± 0.17	1.10 ± 0.10	+ 1	.9

TABLE II

THE WATER CONTENT OF TISSUES REMOVED AT AUTOPSY FROM ALBINO RATS GIVEN AN ACUTELY LETHAL DOSE OF SPIRAMYCIN ORALLY

Tissue	Spiramycin-treated rats $\bar{X}s \pm \sigma$ (% wet wt.)	Control rats $\bar{X}c \pm \sigma$ (% wet wt.)	$\frac{\bar{X}s - \bar{X}c}{\bar{X}c} \times 100$ % wet wt.	% dry wt.	P (difference equals zero)
Carcass	66.43 ± 1.21	69.28 ± 2.45	−4.1	−12.4	.01
Muscle	73.36 ± 3.91	75.53 ± 1.23	−2.9	−11.0	.1
Kidneys	75.22 ± 0.72	76.86 ± 1.27	−2.1	− 8.4	.001
Testicles	84.71 ± 1.77	86.84 ± 2.17	−2.5	−16.1	.05
Stomach	77.19 ± 1.41	78.35 ± 0.77	−1.5	− 6.6	.05
Caecum	78.79 ± 3.43	81.58 ± 2.58	−3.4	−16.3	.05
Colon	76.89 ± 2.69	80.92 ± 1.97	−5.0	−21.5	.001
Small bowel	82.26 ± 1.66	80.98 ± 2.50	+1.6	+ 8.9	.2
Adrenals	72.30 ± 4.37	71.13 ± 2.84	+1.6	+ 6.1	.5
Liver	73.05 ± 1.00	70.95 ± 1.32	+3.0	+11.1	.005

TABLE III

THE WET WEIGHT OF TISSUES REMOVED AT AUTOPSY FROM DOGS GIVEN AN ACUTELY LETHAL DOSE OF SPIRAMYCIN ORALLY

(Wet weight is expressed as percentage of body weight)

Tissue	Spiramycin-treated dogs $\bar{X}s \pm \sigma$	Control dogs $\bar{X}c \pm \sigma$	$\frac{\bar{X}s - \bar{X}c}{\bar{X}c} \times 100$	P (difference equals zero)
Stomach	1.02 ± 0.16	0.723 ± 0.130	+41.1	.01
Small bowel + contents	4.42 ± 1.40	2.77 ± 0.70	+59.6	.05
Colon + contents	0.573 ± 0.045	0.957 ± 0.471	−40.1	.05
Kidneys	0.589 ± 0.060	0.565 ± 0.040	+ 4.2	.4
Liver	3.76 ± 0.66	3.66 ± 0.69	+ 2.7	.8
Adrenals	0.0132 ± 0.0038	0.0125 ± 0.0032	+ 5.6	.7

of normal, particularly in the small intestine, caecum, and colon. There was excessive round cell infiltration in the tunica propria of the caecum. There was a regional necrosis of the hepatic cells and sinusoids of the liver (Fig. 4C), and a regional necrosis of the (mostly distal) convoluted tubules of the kidney (Fig. 4E). Other areas of the liver and kidneys were relatively normal in appearance.

FIG. 4. Photographs of microscopic sections of organs removed at autopsy from albino rats and dogs following death due to an oral lethal dose of spiramycin.

A. A section of the mucosa of the pyloric stomach of an albino rat, showing desquamation of surface epithelium over the gastric foveoles and dilatation of capillaries in the tunica propria. (× 215)

B. A section of the stomach of a dog, showing congestion of blood vessels in the tunica propria and submucosa. (× 50)

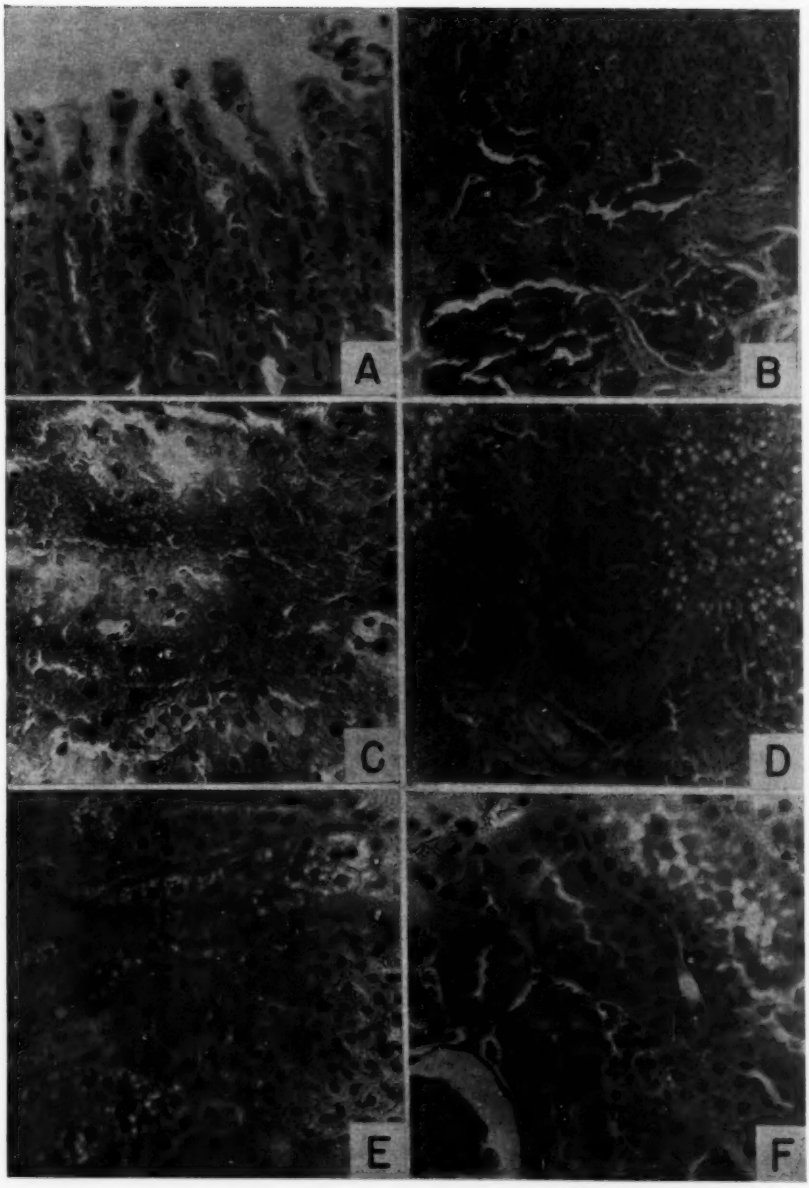
C. A section of the liver of an albino rat, showing necrosis of hepatic cells and sinusoids. (× 215)

D. A section of the liver of a dog, showing a vacuolar degeneration concentrated mainly about the central lobular hepatic vein. (× 50)

E. A section of the kidney of an albino rat, showing necrosis of the (mostly distal) convoluted tubules. (× 215)

F. A section of the kidney of a dog, showing necrosis of the convoluted tubules. (× 215)

PLATE I



Boyd—Can. J. Biochem. Physiol.

B. Dogs

Five dogs died after retaining 6 g. and over of spiramycin per kg. body weight, while nine dogs survived after retaining 4 g. per kg. or less. The LD_{50}, estimated as before, was 5.2 ± 1.6 g. per kg. Clinical effects in the dogs were similar to those described in the rats. Vomiting occurred when the animals were not anaesthetized. At autopsy the stomach was found acutely congested, with an increase in weight (Table III). The stomach was distended with gas, fluid, and unabsorbed spiramycin. The small bowel was markedly dilated and filled with a yellowish fluid. Its total weight (plus contents) was considerably elevated (Table III). The colon, on the other hand, was almost empty and its weight (plus contents, also a yellowish fluid) was reduced (Table III). The water level of the tissues of the gastrointestinal tract was

TABLE IV

THE WATER CONTENT OF TISSUES REMOVED AT AUTOPSY FROM DOGS GIVEN AN ACUTELY LETHAL DOSE OF SPIRAMYCIN ORALLY

Tissue	Spiramycin-treated dogs $\bar{X}s \pm \sigma$ (% wet wt.)	Control dogs $\bar{X}c \pm \sigma$ (% wet wt.)	$\frac{\bar{X}s - \bar{X}c}{\bar{X}c} \times 100$		P (difference equals zero)
			% wet wt.	% dry wt.	
Stomach	76.82 ± 0.99	79.09 ± 1.40	−2.9	−12.4	.005
Small bowel	75.08 ± 3.90	78.79 ± 1.36	−4.7	−18.9	.05
Colon	77.56 ± 0.73	79.27 ± 0.62	−2.2	− 9.6	<.001
Kidneys	77.50 ± 1.19	78.58 ± 0.83	−1.4	− 6.1	.1
Liver	73.08 ± 2.07	72.56 ± 1.26	+0.1	+ 2.7	.6
Muscle	71.94 ± 3.66	70.93 ± 4.12	+1.4	+ 4.9	.7
Adrenals	71.70 ± 4.60	65.24 ± 3.39	+9.9	+35.0	.02

reduced (Table IV). Histological examinations revealed acute congestion of the tunica propria and submucosa of the stomach (Fig. 4B) with desquamation of the columnar epithelium covering the gastric pits. There was an acute, vacuolar degeneration affecting principally the central region of the hepatic lobule (Fig. 4D). There was a tubular necrosis of the kidney (Fig. 4F).

Discussion

The results described above indicate that the acute oral LD_{50} of spiramycin is of the order of 10 g. per kg. body weight in albino rats, and 5 g. per kg. in dogs of 5 to 10 kg. body weight. These results suggest that the oral LD_{50} of of spiramycin is related to surface area of the body and that the oral LD_{50} in man is probably of the order of 1 to 2 g. per kg. body weight. The oral dose of spiramycin recommended for use against moderately severe infections in man is 3 g. per day or approximately 0.05 g. per kg. body weight. This therapeutic dose is 1/20th to 1/40th the estimated oral LD_{50} in man. While these calculations give spiramycin a reasonably safe therapeutic index, some individual persons may be more susceptible than others to the effects of acutely toxic doses of this antibiotic. In particular, the gastralgia, nausea, vomiting, and diarrhea reported in 1 to 3% of patients on spiramycin therapy (8) would

appear to have been due to sensitivity of these patients to the effects of acutely toxic doses. On the other hand, unless the emetic reflex is inactive or inhibited, it is probable that most of a lethal dose of spiramycin taken by mouth would be vomited by man as it is by the dog.

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LIMITATIONS OF THE IN VITRO PITUITARY INCUBATION SYSTEM AS AN ASSAY FOR ACTH-RELEASING ACTIVITY[1]

CLAUDE FORTIER AND DARRELL N. WARD

Abstract

The applicability of Saffran and Schally's *in vitro* pituitary incubation technique to the detection of the ACTH-releasing activity of blood serum fractions, obtained from intact, stressed, adrenalectomized, and stressed–adrenalectomized donor rats, was investigated. The various fractions assayed in this system were found to be markedly active. The activity, which bore no apparent relationship to the physiological state of the donors, was related in part to inhibition of ACTH inactivation, in part to enhanced release of ACTH by the incubated pituitaries. In view of the non-specificity of the observed responses, it is suggested that the increased *in vitro* release of ACTH may reflect passive leakage of the hormone, as opposed to true secretory activity, and the physiological significance of findings obtained with this method is seriously questioned.

In view of recent reports on so-called *ACTH-releasing factors*, based on the utilization of Saffran and Schally's original pituitary incubation system (5, 7, 8, 10), or a modification thereof (11, 12, 13), it was felt that an account of our findings with this method might help to clarify the issue.

Saffran and Schally's technique consists in comparing the amount of ACTH liberated into the medium by rat pituitary glands incubated with or without the principle to be assayed for ACTH-releasing activity (7). The ACTH assay is subsequently carried out by means of another *in vitro* procedure, based on the rate of corticosteroidogenesis of incubated rat adrenal glands (6). The implicit assumption behind this experimental approach is that a significantly increased release of ACTH into the medium corresponds to a higher level of secretory activity by the incubated pituitary gland, induced by a presumably *physiological* activator.

It appeared of interest to test the applicability of this technique to the detection of the ACTH-releasing activity of serum extracts obtained from animals subjected to conditions resulting in elevated blood ACTH levels: adrenalectomy and surgical trauma (14).

Methods

Male Sprague–Dawley rats from the Holtzman Farms, weighing 300–350 g., were utilized throughout this study. Following acclimatization for 2 weeks to our laboratory conditions (constant lighting and temperature (25 ±1°C.); Purina Fox Chow and water *ad libitum*), they were distributed into four groups of 25 animals to be respectively used as intact controls, and subjected to bilateral adrenalectomy, surgical trauma, and surgical trauma 1 week post adrenalectomy.

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Contribution from the Neuroendocrine Unit, Blue Bird Neurological Research Laboratories, Methodist Hospital, and the Department of Physiology, Baylor University College of Medicine (C. F.), and from the Department of Biochemistry, The University of Texas M. D. Anderson Hospital and Tumor Institute (D. N. W.), Texas Medical Center, Houston, Texas. This investigation was supported by a research grant (PHS B-959) from the Institute of Neurological Diseases and Blindness of the National Institutes of Health, Public Health Service.

Bilateral adrenalectomy was performed by the lumbar approach, under ether anesthesia. The animals were maintained postoperatively on a 0.9% NaCl drinking solution. Surgical trauma consisted in exposure to ether for 1 minute, followed by laparotomy and the repeated (six times, each of 10 seconds' duration) crushing of an intestinal loop with a hemostat. The rats were killed by decapitation 2–3.5 minutes after the onset of this procedure, and 1 week after adrenalectomy.

Collection of the Blood

The mixed arteriovenous blood (average yield: 4–5.5 ml. per animal) from the neck vessels was collected into ice-cooled 15 ml. centrifuge tubes and spun for 30 minutes at 3000 r.p.m.

Processing and Fractionation of the Serum

The serum was aspirated with syringe and needle, pooled for each group, and lyophilized to complete dryness. The powder was kept overnight at 0° C. in a vacuum desiccator over a layer of phosphorus pentoxide. On the following day, it was washed three times with dry acetone and lyophilized. The acetone-washed powder was weighed and divided into two equal parts to be respectively fractionated by procedures A and B, aimed at isolating, as a group, the polypeptide and small-protein components of the serum most likely to include the hypothetical ACTH-releasing factor. Residues, precipitates, and soluble fractions were lyophilized to dryness, and stored at 0° C. in a vacuum desiccator over P_2O_5. As a final step, aliquots of the water-soluble fractions of the aqueous methanolic extracts of the untreated (procedure A) or acetic acid-heated (procedure B) acetone-washed serum powder from the group of intact animals were partitioned in an acetone:water system,* and the resulting water-soluble and acetone-soluble extracts were fractionated by paper chromatography, at room temperature, in the ascending system described by Guillemin *et al.* (5) and made up of: acetone, 60 parts; diethylene glycol, 10 parts; 0.5% aqueous urea, 30 parts. The eluates were lyophilized, and the resulting powders stored as above.

Assays

The assays were carried out, with minor modifications, according to the procedure described by Saffran and Schally (7). The adenohypophyses from six donor rats, killed by decapitation, were quickly removed from the skull, separated from the neural lobe, wiped clean of blood, and cut in half. The two halves of each gland were respectively allotted to beakers S and U, containing 1 ml. of Krebs–Ringer–bicarbonate–glucose medium, freshly gassed with 95% O_2; 5% CO_2. Immediately before incubation, 0.1 ml. of an aqueous solution of a 3 ml. serum equivalent† of the extract to be assayed was added to beaker U. Following incubation for 1 hour in a Dubnoff

* The material was dissolved in a minimum volume of water, and three volumes of acetone were slowly added: acetone and water, normally miscible in that proportion, were thus separated into two distinct solvent phases.

† Amount corresponding to the yield of 3 ml. of the original serum.

metabolic shaking incubator, at 38° C. in a water-saturated atmosphere of 95% O_2: 5% CO_2, the incubating media from beakers S and U were aspirated off with a tuberculin syringe, and transferred to small tubes (S and U), containing 0.03 ml. of 1 *N* HCl. The pituitaries were weighed to the nearest 0.005 mg. on an analytical balance, and the pituitary incubating media were neutralized with 0.03 ml. of 1 *N* $NaHCO_3$. Aliquots of 0.150 (U^1) and 0.450 (U^2) ml./100 mg. of adrenal tissue, of the stimulated pituitary medium (U), were run against similar aliquots (S^1 and S^2) of the non-stimulated pituitary medium (S) in Saffran and Schally's 4-point-2 dose *in vitro* assay for ACTH (6). The results, computed and tested for validity by means of Bliss' methods for factorial analysis and analysis of variance (1), were expressed in terms of the relative potency of stimulated to non-stimulated pituitary incubating media,* with confidence limits (antilogs of $C^2M \pm CtSm$) for odds of 19 in 20.

Rationale

Five possibilities may theoretically account for the detection of a significant increase of ACTH in a pituitary incubating medium to which a given principle has been added. It may result from:

1. presence of ACTH in the added principle;
2. potentiation of the response of the incubated adrenals to the ACTH released from the pituitaries (*native* ACTH);
3. inhibition of ACTH inactivation in both pituitary and adrenal incubating systems;
4. enhanced release (whether active or passive) from the incubated pituitaries;
5. a combination of the above.

The following sequence was therefore adopted in an attempt to clarify the mode of action of the extracts under investigation:

A. The extract was added to pituitary-containing medium U, immediately before the incubation, as previously described, and, following incubation, the S and U media were run against each other in the adrenal incubating system. Whenever a positive result ($M^A > 1$)† was recorded, the following complementary experiments were carried out:

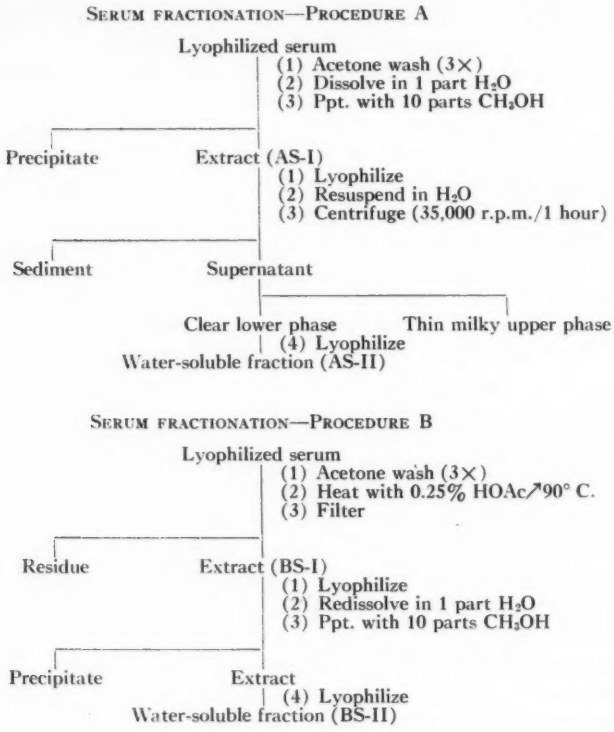
B. The extract was assayed for its ACTH content, by running it directly, in the adrenal incubating system, against U.S.P. Reference Standard Corticotropin (S^1 and S^2), in amounts (U^1 and U^2) corresponding to those used in A.

* The slight difference in the pituitary weights of S and U was taken into account, and, assuming a linear relationship between pituitary weight and response over the range of this weight discrepancy, the values recorded for U^1 and U^2 were multiplied by the following correction factor (CF):

$$CF = (a + bx)/\{a + [\, bx\,(\log U/S)\,]\}.$$

Where a = position of the dosage–response curve at $\bar{x}$, equal numerically to $\bar{y}$ for all dosage levels included in the curve; b = slope of the straight dosage–response line; x = log dose against which the response can be plotted as a straight line; S = weight of the non-stimulated pituitaries; U = weight of the stimulated pituitaries.

† M, which generally designates the log of the potency ratio, is taken here as the antilog. The letters A, B, C, and D, used as exponents of M in M^A, M^B, M^C, and M^D, refer to the correspondingly lettered assaying procedures.



C. The pituitaries were incubated as in A, but the extract was not added to pituitary medium U until after pituitary incubation. The S and U media were then run against each other in the adrenal incubating system.

D. The procedure described in A was followed. However, at the end of the pituitary incubation, an aliquot of the extract corresponding to the amount previously added to U was added to S, the two media being subsequently run against each other as in A and C.

The presence of a detectable amount of ACTH in the extract would be ascertained in B; ($M^B > 0$). A significantly increased potency ratio in C, ($M^C > 1$), would indicate either potentiation of the adrenal response to native ACTH or inhibition of ACTH inactivation. If, following exclusion of the first three possibilities in B and C, the potency ratios were of the same order of magnitude in A and D; ($M^D = M^A$), the excess of ACTH could be imputed, in all likelihood, to enhanced release by the incubated pituitaries. However, a ratio smaller in D than in A; ($M^D < M^A$), observed in conjunction with an elevated ratio in C, ($M^C > 1$), would again point to potentiation of the adrenal response to ACTH, or to inhibition of ACTH inactivation. The inhibition would be exerted during both the pituitary and adrenal

incubating phases of the assay; the potentiation, during the latter phase only. A more pronounced effect, [$(M^A - M^D) > M^C$], would therefore be expected in the first than in the second alternative, [$(M^A - M^D) = M^C$].

Results

As shown in Table I, the AS-II (serum fractionation procedure A) fractions were all markedly active, when tested by assaying procedure A, and, as ascertained in B, none contained detectable amounts of ACTH. In view of the overlapping of the 95% confidence limits of the assays in A, this activity bore no apparent relationship to the prior treatment of the donor animals. That part of the activity was due to increased release of ACTH from the pituitaries is suggested by the elevated potency ratio which, in all cases,

TABLE I

EFFECT OF FRACTIONS AS-II AND BS-II FROM SERUM OF INTACT, ADRENALECTOMIZED, STRESSED, AND STRESSED–ADRENALECTOMIZED DONORS ON ACTH ACTIVITY IN SAFFRAN AND SCHALLY'S *in vitro* SYSTEM

Fraction*	Treatment of donors	3 ml. serum equivalent (mg.)	Assaying procedure†	Potency (antilog M)	95% confidence limits	Index of precision (λ)
AS-II	None	22.8	A	9.84	5.59–26.73	0.071
			B	No ACTH		
			C	2.65	2.14–3.49	0.039
			D	2.42	1.80–3.60	0.050
	Adrenalectomy	29.0	A	20.32	9.08–131.80	0.092
			B	No ACTH		
			C	2.90	1.70–13.49	0.134
			D	5.70	1.55–3.15	0.121
	Surgical trauma	25.4	A	8.85	4.55–107.60	0.130
			B	No ACTH		
			C	2.90	1.70–13.49	0.134
			D	5.70	3.16–35.24	0.121
	Surgical trauma post adrenalectomy	25.5	A	7.38	4.00–28.91	0.095
			B	No ACTH		
			C	1.07	0.67–1.77	0.098
			D	3.95	2.25–16.71	0.119
BS-II	None	30.8	A	4.14	2.36–19.10	0.121
			B	No ACTH		
			C	1.54	1.09–2.38	0.075
			D	5.44	3.92–8.65	0.046
	Adrenalectomy	32.4	A	1.86	1.41–2.68	0.060
			B	No ACTH		
			C	1.14	0.81–1.63	0.072
			D	3.08	1.83–10.67	0.116
	Surgical trauma	29.4	A	0.07	0.01–0.160	0.122
			B	No ACTH		
			C	1.08	0.63–1.92	0.109
			D	6.79	4.34–42.17	0.123
	Surgical trauma post adrenalectomy	26.3	A	5.12	2.86–21.33	0.109
			B	No ACTH		
			C	1.25	0.59–3.53	0.130
			D	2.82	1.75–7.05	0.099

*Water-soluble fractions of the aqueous methanolic extracts of the untreated (AS-II) and of the acetic acid-heated (BS-II) acetone-washed serum powders.

†A: fraction added to medium U, before pituitary incubation; B: fraction assayed for ACTH content against U.S.P. Reference Standard Corticotropin; C: fraction added to medium U, after pituitary incubation; D: fraction added to medium U, before, and to medium S, after, pituitary incubation (see text).

was observed in D, ($M^D > 1$). An elevated ratio in C, ($M^C > 1$), coupled with an A–D ratio difference superior to the ratio observed in C, [($M^A - M^D$) $> M^C$], would further suggest that inhibition of ACTH inactivation was also involved to a considerable extent.

With one exception (BS-II surgical trauma) where inhibition was noted, the BS-II (serum fractionation procedure B) fractions also evidenced a marked degree of activity in A, which bore no relationship to the treatment of the donors and, as shown in B, was not due to contamination with ACTH. Contrary to the AS-II fractions, however, the ratios in C were not elevated ($M^C \approx 1$) and the values in D closely approximated, within the limits of variation of the assays, those recorded in A, ($M^D \approx M^A$). This suggests that an enhanced release of ACTH from the incubated pituitaries mostly accounted for the activity of these fractions, and that potentiation of the adrenal response to ACTH or inhibition of ACTH inactivation were not involved in any appreciable degree.

TABLE II

EFFECT OF CHROMATOGRAPHIC FRACTIONS FROM SERUM OF INTACT DONORS ON ACTH ACTIVITY IN SAFFRAN AND SCHALLY'S *in vitro* SYSTEM*

Starting fraction	Acetone/water partitioned subfraction†	Chromatographic fraction‡	R_f	3 ml. serum equivalent (mg.)	Potency (antilog M)	95% confidence limits	Index of precision (λ)
		α	0.27–0.46	5.3	2.13	1.21–11.80	0.157
	Water-soluble	β	0.46–1.00	4.4	D§		
AS-II		α	0.19–0.46	10.3	4.00	2.29–20.32	0.126
	Acetone-soluble	β	0.46–1.00	4.7	D§		
		α	0.27–0.48	9.1	0.89	0.93–1.36	0.041
	Water-soluble	β	0.48–1.00	9.1	1.84	1.34–3.16	0.076
BS-II		α	0.31–0.65	2.7	1.67	0.82–14.55	0.183
	Acetone-soluble	β	0.65–0.79	7.3	1.79	0.94–9.82	0.164
		γ	0.79–1.00	1.8	2.01	1.16–7.74	0.144

* The assays were restricted to procedure A (see Table I).
† Partition system: acetone, 3 parts/water, 1 part.
‡ Chromatography system: acetone, 60 parts; diethylene glycol, 10 parts; 0.5% aqueous urea, 30 parts.
§ Significant divergence.

TABLE III

EFFECT OF ORGAN EXTRACTS ON ACTH ACTIVITY IN SAFFRAN AND SCHALLY'S *in vitro* SYSTEM*

Organ	Fraction	Aliquot (mg.)	Potency (antilog M)	95% confidence limits	Index of precision (λ)
Liver	AS-II	30.0	D†		
	BS-II	30.0	2.02	1.42–3.36	0.076
Muscle	AS-II	30.0	2.90	2.11–4.63	0.060
	BS-II	30.0	12.22	8.28–20.99	0.041

* The assays were restricted to procedure A (see Table I).
† Significant divergence.

From assays in A of the chromatographic fractions of AS-II and BS-II prepared from the serum of intact donors, it appears (Table II) that the observed activity is not related to any particular group of polypeptides separated by the system utilized. The assays (Table III) of the AS-II and BS-II fractions of liver and muscle tissue (prepared in the same way as their serum homologues) further point to the nonspecificity of the observed effects.

Discussion

Saffran and Schally's procedure for assaying ACTH (6) has been extensively used in this laboratory over the last two years. It has proved both convenient and reliable. Being based on the actual rate of corticosteroidogenesis, it provides a more direct indication of adrenal cortical activity than Sayers' adrenal ascorbic acid depletion test (9). It is highly specific for ACTH; of numerous substances tested, none but ACTH or pituitary extracts has been found to increase the rate of corticosteroidogenesis (3). As an additional advantage, it requires but 20% of the time necessary to carry out an assay by Sayers' procedure. It is more accurate than the latter and offers a wider useful range (rectilinear portion of the log dose response slope; this range extends from 3 to 300 milliunits/100 mg. of adrenal tissue in Saffran and Schally's assay, as compared to 0.15 to 2.5 milliunits/100 g. B. W. in Sayers').

It is felt, by contrast, that results based on the rate of ACTH release by the incubated pituitary should be interpreted with extreme caution. As distinct from the adrenal cortex, which does not store its secretory products to any appreciable extent (the small amount of steroids present in the gland is reportedly flushed out during the 1-hour preincubation period which precedes the addition to the system of the substance to be assayed (2)), the adenohypophysis is a storage gland. It follows that, whereas the rate of steroid release from the incubated adrenal truly corresponds to its secretory activity, the liberation of ACTH from the pituitary may simply reflect passive leakage of the hormone. Moreover, whereas steroids are stable compounds, ACTH is readily inactivated by incubation (4).

Admittedly, the fractions considered in this study were assayed at a relatively high dosage level.* This was necessitated by the nature of our source material, blood serum. According to Sydnor and Sayers, the concentration of ACTH in the peripheral blood of the intact rat is less than 0.5 milliunit (0.5 μg. of the original La-1-A standard)/100 ml., and beyond the limits of sensitivity of their method, when an oxycellulose adsorbate of a 30 ml. blood equivalent is injected per assay rat (14). A *physiological* ACTH-releasing principle, were it detectable at all in the blood of the intact or stressed rat, would be presumably present in much smaller amount. Hence the tentative choice of a 3 ml. serum equivalent as the minimal aliquot to be assayed initially.

* It should be pointed out, however, that the chromatographic fractions of AS-II and BS-II were assayed, in terms of weight/volume, at a level approximating the doses of fresh tissue extracts used by Saffran and Schally in their original studies (7).

Our results are proof that the pituitary incubating system is not applicable to our purpose. They raise additional doubts, moreover, as to the physiological significance of findings based on the release of ACTH from the incubated pituitary.

Two essential prerequisites would be needed to substantiate the claim that a principle, active in Saffran and Schally's *in vitro* system, is the mediator of the pituitary–adrenocortical activation induced by stress:

1. The elucidation of its mode of action (whether it involves release of ACTH, contamination with ACTH, potentiation of its effects, or inhibition of its inactivation).

2. The demonstration that the increased release, if ascertained, corresponds to enhanced secretory activity of the pituitary, as opposed to passive leakage of the hormone through its cells. This could be indirectly achieved by showing that the principle considered activates the pituitary–adrenocortical axis in an otherwise unresponsive *in vivo* preparation, such as the hypothalamic-lesioned or hydrocortisone-inhibited animal.

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THE ROLE OF THE SALIVARY DUCT SYSTEM IN THE FORMATION OF THE SALIVA[1]

A. S. V. Burgen and P. Seeman

Abstract

A method is described for testing whether the saliva changes in composition as it passes down the secretory ducts. The use of radioactive potassium, sodium, and urea shows an exchange of these materials with the blood after secretion by the acini. Analysis of the relationships between specific activity of the isotope in the saliva and venous blood leads to the conclusion that the exchange is occurring between the saliva in the ducts and blood flowing in a capillary system surrounding the ducts. In these capillaries the blood is flowing in a direction counter to that of the saliva. A mathematical analysis of the behavior of urea in the ducts has been made. A theory of salivary secretion is presented in which the primary secretory event is the extrusion of potassium and accompanying anions into the acini. Water follows the ions passively. In the ducts the potassium is reabsorbed and only partly replaced by sodium ions diffusing in from the plasma. The potassium is recycled to the acini by the tubule portal system.

Introduction

The relationships between the concentration at which solutes are secreted in the saliva and the rate at which the saliva is secreted are complicated. Two types of explanation have been offered to explain them. In the first explanation all the complex segregation of solutes has been attributed to the acinar cells and the ducts have been regarded as impermeable conduits carrying the acinar saliva to the exterior without appreciable change in composition. In the second scheme the elaboration of a primary secretion by the acinar cells is postulated, followed by modification of this secretion in the duct system. This second scheme was suggested early in the history of the study of salivary secretion by Merkel, Werther, and others (1, 2, 3) because of the histological appearance of the ducts. The epithelium of the ducts seemed to them to have a degree of histological complexity that was excessive for a mere conduit, and under some conditions appearances suggestive of the extrusion of secretion into the lumen were found (4, 39). Merkel (1) specifically attributed the function of secretion of calcium to the columnar duct cells as a result of their staining reaction with pyrogallol. More recently it has been suggested that the primary secretion of the acini is isotonic and rich in sodium and that the hypotonicity of the saliva is due to sodium reabsorption in the ducts (5, 6). This theory rests on no secure experimental basis; indeed, up to the present, the only conclusive evidence in favor of any role played by the ducts in salivary secretion has been the demonstration by radioautographic and kinetic methods (7, 8, 9) that iodide and thiocyanate are actively transferred into the saliva through the ducts and not through the acini.

We have been led to the reconsideration of the role of the ducts in salivary secretion presented in this paper as a result of experiments designed to eluci-

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date the nature of the time dependence of the potassium secretion in the saliva (10). In this work it was found that in the submaxillary gland of the dog when secretion was produced by chorda tympani stimulation after a period of rest, the saliva secreted initially had a high potassium concentration. This high concentration occurred only during the first few minutes and thereafter the potassium concentration in the saliva reached a steady level 1.5–3 times the arterial plasma level. Synchronous with these changes in saliva potassium concentration, there were parallel changes in the potassium level in the plasma of the venous effluent from the gland. Immediately after stimulation started the venous plasma potassium reached two to three times the arterial plasma level, and this was succeeded by a steady level in the venous plasma one-fourth to one-half of the arterial plasma concentration.

The time courses of the transient potassium concentration in the saliva and venous plasma were very similar and the amounts of potassium lost from the gland in the two transients bore a close relationship to each other over a wide range of rates of saliva secretion. The initial loss of potassium from the gland into both saliva and venous blood led to a rapid depletion of the gland potassium.

This phenomenon can be explained in two ways, which, it will be seen later, really accord with the two general theories of secretion stated above. On the one hand, it could be that activation of the acinar cells by the parasympathetic nerves leads to a general reduction of the electrochemical potential for potassium in the cells (or an increase in outward permeability) so that potassium would be lost from the cells both across the face opposed to the acinar lumen and that opposed to the extracellular fluid. This explanation goes against the generally held view that secreting cells are functionally polarized so as to produce a unidirectional transfer. However, this general concept does not invalidate the explanation. The second alternative explanation is that while the acinar cells transfer potassium only in the general direction extracellular fluid → saliva, a back movement of potassium from saliva into the blood can also occur. Such a movement might occur through the interspaces between the acinar cells or across the duct walls.

The difference between these two explanations really resolves itself into the question of whether there is a primary secretion differing from the external secretion or not and may be studied experimentally in the following way, using isotopically labelled substances. If a radioactive substance is secreted by the acini and undergoes no modification before reaching the exterior, then its specific activity in the secreted saliva must always be identical with that in the isotope pool from which it is derived, i.e. the intracellular pool of the gland. On the other hand, if any subsequent modification of the solute concentration occurs either by intercellular or transductal movement a partial equilibration will occur between the specific activity of the isotope in the saliva and that in the extracellular fluid. In order to detect these changes the specific activity of the isotope in the acinar cells must be different from that in the extracellular fluid. The specific activity of the plasma (and hence

extracellular fluid) will be higher than that in the cells if the gland is gaining isotope (this is referred to as a loading type of experiment) and in this case postacinar equilibration will be demonstrated if the saliva specific activity is higher than that in the cells. Conversely the specific activity in the extracellular fluid will be lower than that in the cell water if the gland is losing isotope (this is referred to as an unloading type of experiment) and in this case, postacinar equilibration will lead to a specific activity in the saliva that is lower than that in the cells. This paper describes experiments with isotopically labelled potassium, sodium, and urea, and presents an analysis of the processes that probably account for the results obtained.

Experimental

All experiments were carried out on adult mongrel dogs anaesthetized with chloralose and urethane.

Loading Experiments

These could only be carried out successfully with radioactive potassium, since only in this case was the intracellular pool large enough to permit a reasonable duration of experiment.

The experimental setup was identical with that used for potassium balance (10) but in addition an intravenous infusion of K^{42} as KCl was given. In different experiments this was arranged to give (*a*) a rectangular step of K^{42} concentration in the arterial blood, (*b*) a steadily rising concentration, or (*c*) an exponentially falling concentration.

Unloading Experiments

These were of more general application to the study of isotope exchange in the secretion but needed a more extensive dissection in which all the branches of the carotid artery other than the one or two arteries going to the gland were ligated. The small accessory artery to the gland that enters on the dorsolateral aspect was ligated if present. The dissection was continued until the gland had been completely freed from its bed and was attached to the animal only by its artery and veins. At this stage as much as possible of the sublingual gland was removed, the submaxillary gland was then replaced *in situ*, covered with pads soaked in warm saline, and the skin closed over it. This preparation is similar to that described by Gibbs and Szeloczy (11). In ligating the arteries, care was taken to ligate them close to the carotid to avoid pockets in which isotope could form a reservoir, and the only artery that was ligated as far as possible from the carotid was the lingual. The central end of the lingual artery was cannulated and connected to a motor driven syringe for infusion of the isotope.

After collection of the venous effluent had begun, the gland was loaded with isotope by a slow infusion into the central end of the lingual artery.

Adequate loading occurred after 5–10 minutes for Na^{22} and C^{14}-urea but 25–40 minutes was necessary with K^{42}. When the loading period was complete, blood was refluxed up the arterial cannula to avoid producing a small

pocket of isotope in the lingual artery stump. Since all the effluent from the gland was collected, *no* isotope reached the general circulation and after the loading period was completed, the gland was being autoperfused with the animal's own unlabelled blood. Usually 2–5 mg./kg. heparin was given intravenously after all the dissection was completed, and the blood loss from the animal was replaced by periodic intravenous injections of 50 ml. of fresh heparinized dog blood.

The infusion solutions were made up in fresh dog plasma to which approximately 1 mg. of T1824 dye was added as a marker.

K^{42} was obtained from Atomic Energy of Canada as K_2CO_3 with an activity of 5–10 mc./g.

Na^{22} as NaCl with an activity of 25 mc./g. and C^{14}-urea with an activity of 15 mc./g. were obtained from the Radiochemical Centre, Amersham, England.

The gland was loaded with 10–20 μc. of Na^{22} or C^{14}-urea and 1–2 μc. of K^{42}. The smaller amount of K^{42} needed was due to the greater proportion picked up by the gland and the higher counting efficiency for this isotope.

Estimation of Radioactivity

Counting was done with a thin mica window GM tube (Tracerlab TGC2). For K^{42} and Na^{22} estimation the plasma was centrifuged off the cells immediately after collection and 0.2 ml. plated directly with addition of 1 drop of 10% Carbowax 4000 to minimize flaking. Under these conditions no appreciable loss of labelled Na or K occurred through penetration into the red cells. The saliva was plated directly. At the end of the experiment the gland was removed, weighed, and then digested in concentrated nitric acid. Aliquots were plated and counted. No corrections for self-absorption were necessary for K^{42}, but a small correction was necessary for Na^{22}. Plasma, saliva, and gland C^{14} were estimated after deproteinization with barium hydroxide – zinc sulphate (12). The filtrate was then plated directly. Correction for self-absorption amounted to 10–15% and standard curves were prepared for filtrates from plasma, saliva, and the gland, and the appropriate correction was applied.

Estimation of Na, K, and Urea

Na and K were estimated by flame photometry as described previously (10). Intracellular concentrations were calculated from measurements of extracellular space made with inulin on other glands that had been similarly treated (Burgen unpublished). The mean extracellular water was 19.1% of the gland weight. Urea was measured by the urease – Nessler method of van Slyke and Plazin (13) as modified by King (14). Controls with urease added in reverse order were made so that correction could be made for ammonia present in the samples. To minimize this correction the blood urea was raised by the intravenous injection of 25–50 ml. of 20% urea solution 2 hours before the isotope was given. Finally, in some experiments with urea, suppression of urea-splitting organisms in the alimentary canal was attempted by preliminary administration of sulphadiazine and streptomycin (15). This did not modify the results.

Results

Potassium

With potassium the most useful data were obtained in the unloading type of experiment. Fig. 1 shows the loss rate of K^{42} in the venous blood and saliva in a typical experiment. In the initial resting period the loss rate fitted a simple exponential curve. At the end of the rest period K^{42} was being lost at a rate of 2100 c.p.m./g. minute. With the onset of stimulation at 5 cycles per second the loss rate rose abruptly to 45,000 c.p.m./g. minute and then gradually declined, at the end of stimulation an abrupt drop of K^{42} loss rate occurred and thereafter a gradual return towards the initial state was found. The output of K^{42} in the saliva was greater than that in the venous blood and the fall off in output through both routes ran parallel.

It was possible to compute the gland content of K^{42} at any time during the experiment from the data obtained by gland analysis at the end of the experiment, and integration of the total counts lost in the blood and saliva during the course of the experiment. Fig. 2 shows the result of such a calculation of gland K^{42} content. In the initial resting period the rate of K^{42} loss from the gland was 0.0102 per minute (log slope constant). In the first minute of stimulation the loss rate increased about 40-fold to 0.40 per minute and for the whole 5 minute period the loss rate averaged 0.10 per minute. The resting exchange rate in this experiment corresponds to a potassium flux of 0.77 μeq./g. minute.

Since our continuous balance type of experiment also enables us to calculate the total potassium of the gland at any time we can also calculate the specific activity of K^{42} in the gland (specific activity is the ratio of radioactive isotope to that of all isotopes of the substance concerned, and is proportional to c.p.m./μeq. of the substance).

In making this calculation we assume that all the potassium in the gland is exchangeable; it will be shown later in this paper that this appears to be the case. Were some of the potassium inexchangeable our estimates of specific activity in the cells would be too low.

If we replot the data of Figs. 1 and 2 in terms of specific activity we get the results shown in Figs. 3 and 4. Considering first the venous specific activity (S_v), this stayed fairly constant at about 70% of the intracellular activity (S_c) in the resting state. With the onset of stimulation S_v rose sharply to near the intracellular level and then rapidly fell away to S_v/S_c = 0.3–0.4. After stimulation was over the venous specific activity ratio returned towards the initial resting value.

The specific activity of potassium in the first few drops of saliva secreted was almost as high as in the intracellular potassium, but the S_s/S_c ratio gradually fell to a value of 0.77. Table I shows the steady values reached in six experiments in which S_s/S_c ranged from 0.45–0.91 and S_v/S_c from 0.35–0.95.

These values are all significantly less than 1.0 but the differences are not large. However, the following consideration shows that these values are of

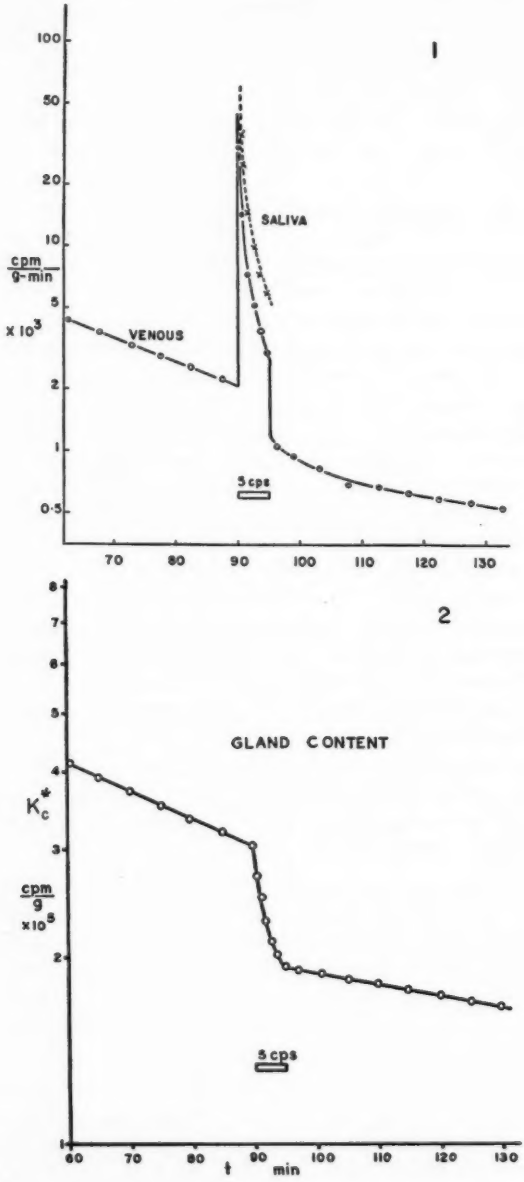

FIG. 1. Unloading experiment with K^{42}. Loss rate of radioactivity in venous blood and saliva. Chorda tympani stimulated maximally at 5 cycles per second for 5 minutes.
FIG. 2. Same experiment as Fig. 1. Gland content of K^{42} calculated. Note rapid loss of K^{42} from the gland during stimulation.

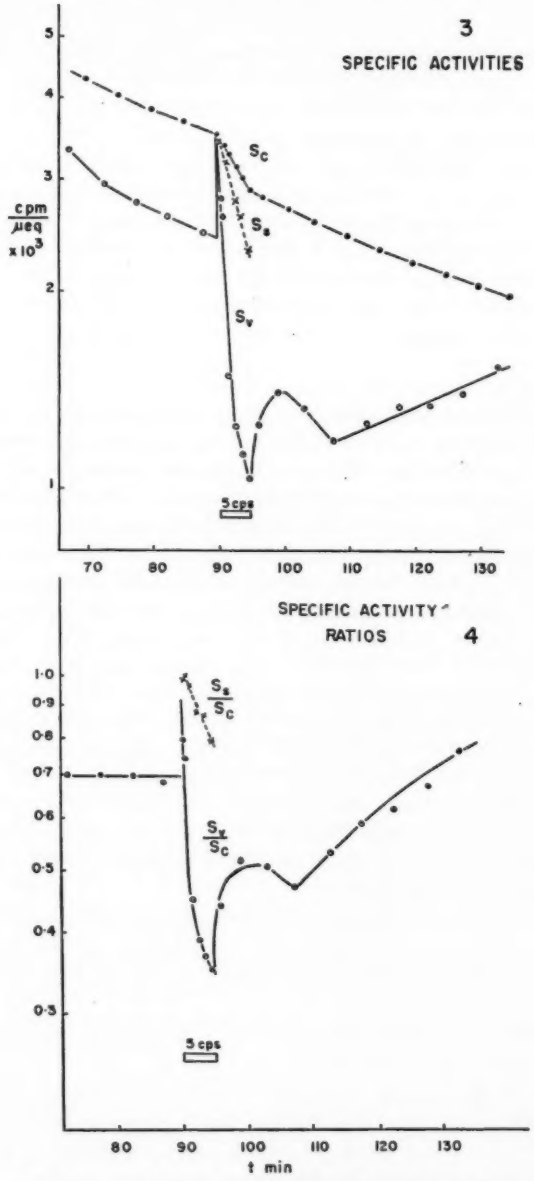

FIG. 3. Same experiment as Fig. 1. Calculated as specific activities in 10^3 c.p.m./μeq. K. S_c = specific activity in the cell potassium; S_s = specific activity in saliva potassium; S_v = specific activity in venous plasma potassium.

FIG. 4. Same experiment as Fig. 3. Specific activities calculated as ratios to that within the cells.

TABLE I

RADIOACTIVE POTASSIUM EXCHANGE IN THE SUBMAXILLARY GLAND MEASURED BY THE UNLOADING METHOD

Stimulation frequency, cyc./sec.	Saliva flow, g./g. min.	S_s/S_c	S_v/S_c	S_s/S_v
20	0.153	0.80	0.80	1.00
7	0.102	0.72	0.66	1.09
5	0.208	0.79	0.35	2.26
3	0.105	0.84	0.63	1.32
2	0.051	0.91	0.77	1.18
Spontaneous secretion	0.0014	0.45	0.95	0.47

the order to be anticipated and that very low values of S_s/S_c cannot be expected. The limiting value of S_s/S_c depends on the size of the potassium pools that are equilibrating. The arterial plasma contains only 4 meq./l. of potassium whereas the saliva contains 8–15 meq./l. Further, if a net movement of potassium from the primary secretion into the blood is occurring the concentration of potassium in the primary secretion must be a good deal higher than this. Thus, making allowance for the greater magnitude of plasma than of saliva flow, we can estimate that the saliva potassium pool is at least as large as and probably larger than the plasma pool. Hence even if complete equilibration between saliva and plasma potassium were to occur S_s/S_c would be greater than 0.5 at all rates of secretion, except the very slowest in which the plasma pool size is more favorable. We would also expect the dilution of specific activity to be less at the beginning of stimulation for two reasons. First, the saliva potassium is highest and hence the saliva pool size is greatest at this time, and second the increase in blood flow at the beginning of stimulation always lags slightly behind the secretion of saliva (10). Both these factors will make the saliva pool size initially considerably greater than the plasma pool size.

It will be noted that in several of the experiments the specific activity in the venous plasma was very similar to that in the saliva, and in the experiment in which a very slow spontaneous secretion occurred it was greater than in the saliva.

This effect will be discussed later and is of considerable importance for the interpretation of the results described.

Experiments of the loading type gave similar evidence suggesting that the secondary equilibration theory is correct, but the same limitations due to the size of the saliva and plasma potassium pools prevented a decisive proof of the theory. More prolonged loading experiments were made to see whether the gland potassium was completely exchangeable.

In two experiments after 4 hours the glands reached 90 and 98% of the specific activity of the arterial plasma (with an exchange constant of 0.01 per

minute the predicted exchange at this time is 91%). After 24 hours equilibration two experiments gave 91% and 105% of the arterial plasma level. In shorter term experiments in which the internal specific activity was estimated by balance, a close proportionality was maintained between K^{42} uptake and the transcellular K^{42} gradient. This proportionality was unaffected by the degree of loading and this and the other data suggested that at least 90% of the gland potassium was exchangeable and that to a first approximation its exchange could be expressed by a single exponential rate constant.

In the loading experiments the uptake of K^{42} was greatly accelerated when the gland was secreting, such that the intracellular specific activity came into equilibrium with the arterial plasma within 10 minutes. Evidently potassium uptake as well as potassium loss was stimulated by excitation of the parasympathetic nerves.

The results up to this point were suggestive that secondary equilibration of potassium occurs after secretion by the acinar cells, but were not sufficiently convincing to firmly establish its occurrence.

However, it seemed not improbable that if potassium ions were able to equilibrate with the extracellular fluid across some structure in the glands, then other substances of similar molecular dimensions might also be able to traverse the same path. If these substances were free from the limitations that pool size imposes on potassium a more decisive result might be obtainable. Urea and sodium were finally chosen as most nearly meeting these requirements.

Urea

Previous work with urea (16) has shown that it is treated passively in the salivary gland as judged by the criterion that the concentration ratio between saliva and plasma (S/P ratio) is independent of the absolute plasma urea concentration. In addition the concentration of urea in the cell water of the gland is the same as that in the plasma water. At the rates of saliva secretion that are convenient experimentally (0.05–0.4 ml./g. minute), the saliva urea is 40–60% of the plasma level, with the over-all concentration gradient therefore being in the direction of blood to saliva in distinction to that for potassium.

The diffusion of urea into erythrocytes is so fast (17) that complete equilibration can be considered to occur in the time the blood is present in the gland capillaries; the intravascular pool is therefore in the whole blood water rather than just the plasma.

Fig. 5 shows a typical experiment with urea. Initially the C^{14}- urea was leaving the gland at a rate of 0.12 per minute and at the beginning of stimulation the loss rate rose to 0.65 per minute. The form of the curve was quite similar to those obtained with K^{42}, but the amount of radioactivity lost in the saliva was considerably less than that lost into the blood. Fig. 6 shows the results of this experiment calculated as specific activities. At rest the ratio S_v/S_c was 0.17, and with stimulation the specific activity ratio in the venous blood fell and reached a level of about 0.055. The specific activity

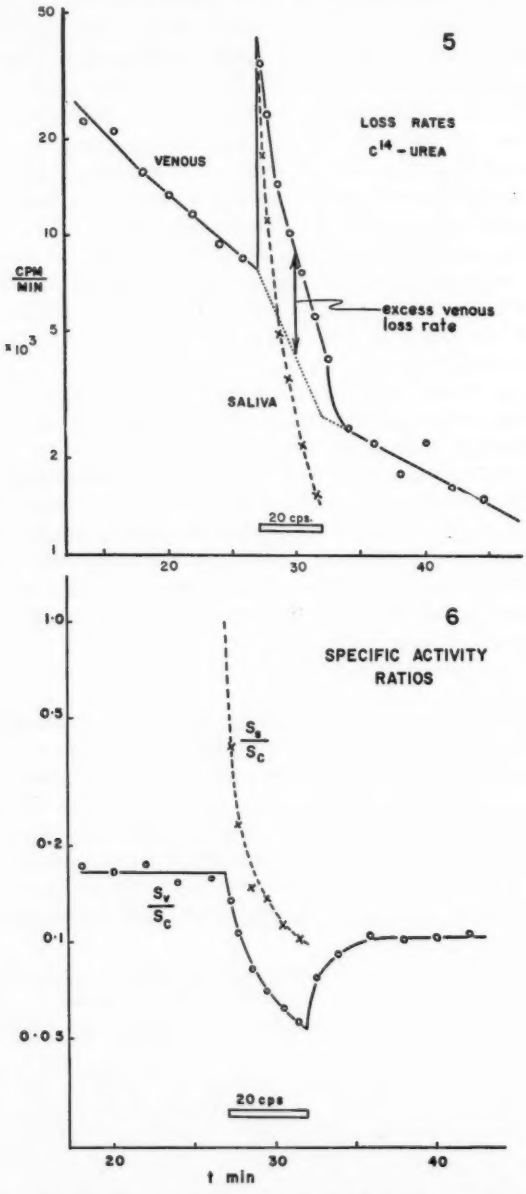

FIG. 5. Unloading experiment with C^{14}-urea. Loss rate of radioactivity in venous blood and saliva. Chorda tympani stimulated maximally at 20 cycles per second for 5 minutes. Method of obtaining the "excess" venous loss rate during the stimulation is indicated.

FIG. 6. Same experiment as Fig. 5. Specific activity ratios.

TABLE II

RADIOACTIVE UREA EXCHANGE IN THE SUBMAXILLARY GLAND MEASURED BY THE UNLOADING METHOD

Stimulation frequency, cyc./sec.	Saliva flow, g./g. min.	S_s/S_c	S_v/S_c	S_s/S_v
20	0.341	0.10	0.056	1.79
20	0.318	0.10	0.060	1.67
20	0.140	0.15	0.066	2.25
6	0.185	0.17	0.082	2.08
2.5	0.101	0.11	0.074	1.52
1	0.090	0.5	0.25	2.00
0.5	0.041	0.11	0.17	0.65
0.4	0.041	0.28	0.25	1.12

ratio in the saliva was less than one even at the beginning of stimulation and it rapidly fell off to a value of slightly less than 0.1. In a series of eight such experiments very similar results were obtained (Table II).

The results were perfectly consistent and provide strong support for the secondary equilibration hypothesis. The slowness with which equilibration was reached was somewhat surprising in this case. With K^{42}, as pointed out previously, this can be regarded as a natural consequence of the delayed increase in blood flow and the character of the potassium transient in the saliva. It is possible that the urea permeability is to some extent conditioned by the potassium concentration in the duct lumen. With urea as with potassium the specific activity in the venous blood may exceed that in the saliva at low rates of secretion. An example of such an experiment is shown in Fig. 7 (see also Table II).

Sodium

Unloading experiments with Na^{22} gave very similar output curves to those obtained with urea and potassium but the exchange rate was even faster. In the experiment of Fig. 8 the resting Na^{22} exchange was 0.21 per minute, corresponding to a resting sodium flux of $\sim$ 1 μeq./g. minute. These rapid exchanges required that a rather large correction factor for the exchange between the gland extracellular fluid and the plasma and also for the dead space of the veins and venous cannula be applied to the calculation of intracellular specific activity. In addition, because the intracellular sodium was very low, its determination involved a considerable error. Taken together these factors made calculation of the specific activity of the intracellular sodium subject to a large error so that the results for sodium must be considered as giving the order of the results only and little confidence can be placed in quantitative deductions drawn from them. However, the saliva specific activity ratios (Fig. 9) were so low that the qualitative significance of the results cannot be in doubt. In this case S_s/S_c fell to 0.034 and, in other experiments, values of 0.004–0.143 were obtained (Table III). Indeed, despite the difficulties mentioned the reproducibility of the results was very

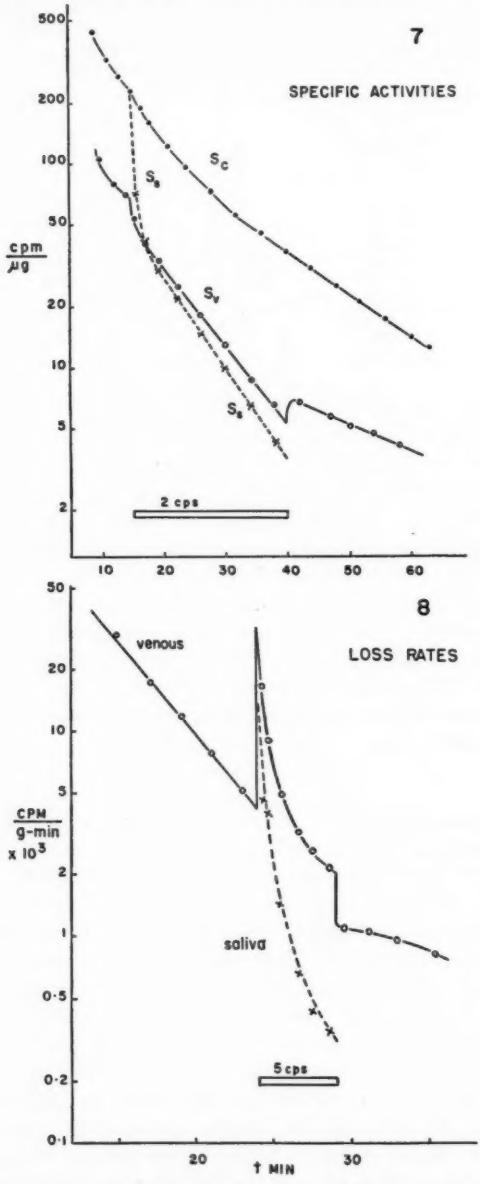

FIG. 7. Unloading experiment with C^{14}-urea. Specific activities in the cells, saliva, and venous blood water. Chorda tympani stimulation at 2 cycles per second for 25 minutes. This figure is included to show an experiment in which the saliva specific activity (S_s) was consistently lower than the venous specific activity (S_v).

FIG. 8. Unloading experiment with Na^{22}. Loss rate of radioactivity in venous blood and saliva. Chorda tympani stimulation at 5 cycles per second for 5 minutes.

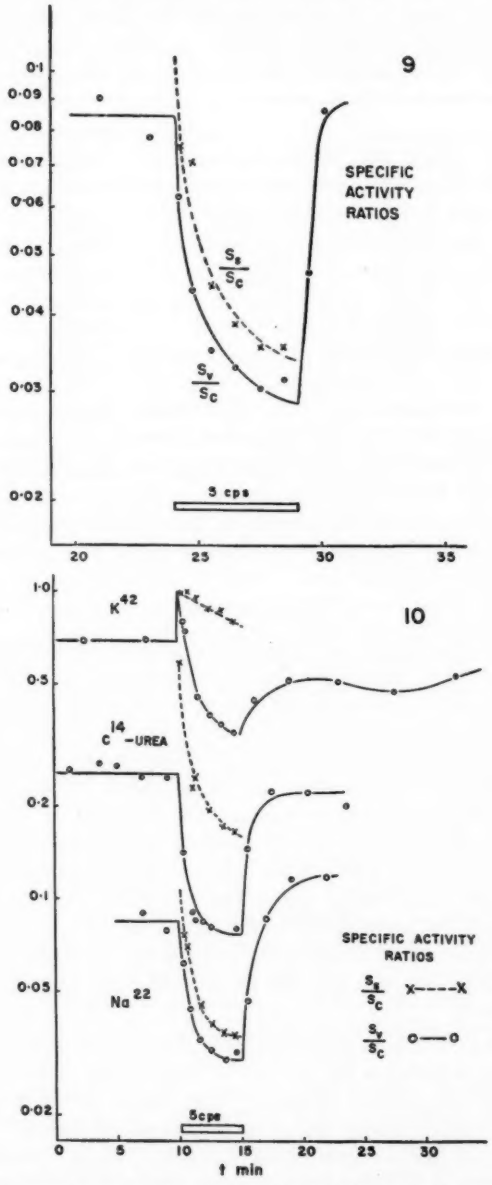

FIG. 9. Same experiment as Fig. 8. Specific activity ratios in venous plasma and saliva.

FIG. 10. A comparison of the specific activity ratios found with K^{42}, C^{14}-urea, and Na^{22} in three comparable experiments in which stimulation was at 5 cycles per second for 5 minutes.

TABLE III

RADIOACTIVE SODIUM EXCHANGE IN THE SUBMAXILLARY GLAND MEASURED BY THE UNLOADING METHOD

Stimulation frequency, cyc./sec.	Saliva flow, g./g. min.	S_s/S_c	S_v/S_c	S_s/S_v
20	0.175	0.143	0.074	1.93
20	0.195	0.088	0.084	1.05
10	0.241	0.004	0.005	0.80
10	0.125	0.074	0.047	1.57
5	0.126	0.071	0.065	1.09
5	0.134	0.137	0.140	0.98
5	0.210	0.035	0.030	1.17
2	0.017	0.074	0.150	0.49

reassuring. We can regard the results with sodium as providing further proof that extensive postacinar equilibration of a primary secretion occurs. With sodium, as with potassium and urea, values of S_v greater than those for S_s sometimes occurred (Table III).

Fig. 10 gives specific activity curves for comparable experiments with the three test substances. The qualitative similarity of the results can be seen.

Discussion

The results presented argue strongly in favor of secondary equilibration of urea and sodium after they have been secreted by the acinar cells, and it does not seem unreasonable to regard this evidence as so reinforcing the more dubious data on potassium as to make it highly probable that potassium also behaves in this way. We would thus regard the potassium transient observed in the venous effluent from the gland as due to a back diffusion into the blood of a fraction of the potassium secreted by the acinar cells into the primary secretion. It is self-evident that any area of the gland permitting isotope exchange between the primary saliva and the blood and also permitting a net potassium movement out of the saliva will probably play a dominating role in determining the composition of the saliva secreted externally and it is therefore desirable to examine our data in more detail to see if we can reach any conclusions as to the site and the quantitative features of the secondary equilibration process.

The key to this problem is, we believe, the observation that in some experiments with all three isotopes the specific activity in the saliva fell below the specific activity in the venous effluent. This could not occur if the dilution of specific activity in the saliva were due to interstitial diffusion of the solute into a network of capillaries surrounding the acini. We can, however, account for this phenomenon if equilibration occurred across the duct epithelium.

If the equilibration occurs across the duct wall a large blood flow to the ducts is required to provide a large enough plasma pool of unlabelled solute to dilute the isotope. This blood might be provided either (*a*) as a separate

circulation from that supplying the acini or (*b*) as a series system of capillaries in which a continuous capillary network supplies both ducts and acini in a manner analogous to the glomerulotubular perfusion system in the mammalian kidney.

Let us first examine the possibility of a separate vascular bed for the ducts as opposed to the acini. We have shown (9) that iodide is secreted by the duct system of the parotid gland and that the magnitude of the iodide clearance leads to very high blood flows for the duct system of this gland; indeed they are of the order of the estimated total gland blood flow. A similar order of duct flow would be needed to convey the potassium transient into the venous blood. Yet at rest the specific activity of the venous blood potassium can come within a few per cent of the intracellular specific activity of the acinar cells. The only blood that can come into equilibrium with the acinar cells *at rest* is that in contact with them — this means that nearly all the resting blood flow perfuses the acini. The only way that most of the blood can perfuse *both* acini and ducts is through a series capillary system. It is not possible to be as certain that such a high proportion of the blood flow occurs through a series vascular system during activity because, although under these circumstances iodide clearances indicate that the duct blood flow rises in proportion to the total blood flow, potassium equilibration is no longer valid as a measure of acinar perfusion rate. However, the venous plasma potassium may fall to as little as one-quarter of the concentration in the arterial plasma. A minimum of three-quarters of the total blood flow would need to perfuse the acini to permit this, even if one assumes that the acini can completely clear the perfusing blood of potassium, under which conditions all the venous potassium would be contributed by blood not perfusing the acini. Consideration of these arguments shows the strong likelihood that during activity of the gland a large proportion of the gland blood flow runs in an acinoductal portal system.

The portal system may be one in which the terminal arteriole breaks up into capillaries around the duct; these then run from its distal to its proximal end, there to become continuous with the capillaries around the acini. The acinar capillaries are tributary to the gland veins. In this system the general direction of blood flow in the periductal capillaries is in the opposite direction to that of the saliva. This may be called a countercurrent portal system. The other type of portal system is one in which the direction of blood flow is the same as that of the saliva, the blood perfusing the acini before the ducts. This may be called a concurrent portal system.

Fig. 11 summarizes diagrammatically the specific activity relationships in these systems. Fig. 11 (*a*) shows that the equilibration across the duct wall in the countercurrent system is between the saliva and the arterial blood, and if the duct permeability is sufficiently great the specific activity in the saliva will approach that in the arterial blood (i.e. zero specific activity in an unloading experiment). On the other hand, in the concurrent system shown in Fig. 11 (*b*) when the duct permeability is very great the limiting value will

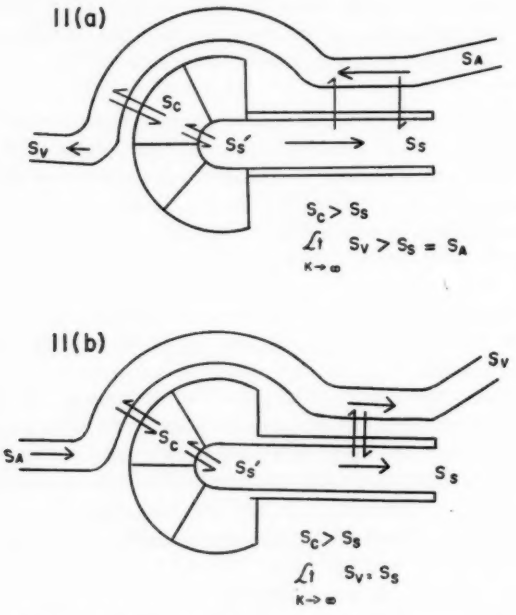

FIG. 11. A simplified diagram of the two types of acinoductal blood perfusion.
(*a*) (Upper diagram.) The countercurrent system. Transductal exchange occurs between material in the saliva and the arterial inflow.
(*b*) (Lower diagram.) The concurrent system. Transductal exchange occurs between material in the saliva and the venous outflow.

be an approach of saliva specific activity to that in the venous plasma. In this case it is not possible for the saliva specific activity to fall below that in the venous blood. The results obtained with all three isotopes show that it is possible for the saliva specific activity to fall below that in the venous blood. We are therefore led to the conclusion that the portal system in the salivary glands must be a countercurrent system.

There is anatomical evidence that the ducts have a rich blood supply (18, 19, 20). Kowalewsky (18) made injection preparations of the cat's submaxillary gland and found that when the injection mass was perfused into the artery to the gland it filled a rich network of vessels around the duct system at a stage when little medium had entered the vessels surrounding the acini; venous injection usually filled the acinar vessels more satisfactorily. Kowalewsky's preparations also suggest that *some* arterial blood may go directly to the acini. Our evidence suggests that this may be only a small fraction of the total gland flow. From Kowalewsky's illustrations it is not possible to be quite certain that he had shown the presence of the portal system as we have illustrated it. Zimmermann (19) and Flint (20) both pointed out that the arteries to

the gland run along the ducts and ramify with the ducts and hence are countercurrent, but they did not extend their study to the smallest ducts and the capillaries. In Maximow and Bloom's textbook (21) a countercurrent capillary net in the pancreas is illustrated but its provenance is not stated.

Holtzlohner and Niessing (22, 23) studied the circulation in the cat submaxillary gland by direct observation. Their observations led to the postulation of a system of arteriovenous anastomoses. These are not compatible with the data discussed above. Brucke and Zwiauer (24) repeated these experiments and were unable to confirm Holtzlohner and Niessing's results.

It is highly desirable that a more satisfactory study of the gland circulation should be made, but in the absence of such a detailed study we will assume that the countercurrent portal system as described above is the predominant circulatory pattern in the submaxillary gland.

If we can assume that the duct segment across which equilibration of solutes takes place is reasonably uniform we can develop a quantitative treatment of the duct transfer process that should be adequate for a substance like urea that behaves passively, and for which the transfer can reasonably be attributed to thermal diffusion along a chemical potential gradient. The analogous problem of heat transfer in a countercurrent heat exchanger is treated by Fishenden and Saunders (25) and their equations with the appropriate substitutions give

$$[1] \qquad C = C_0 - \frac{V'}{V+V'}(C_0 - C_0')\left[1 - \exp\left\{-\left(\frac{1}{V}+\frac{1}{V'}\right)kx\right\}\right],$$

an equation describing the concentration change in the saliva as it proceeds along the duct segment responsible for equilibration from its acinar end (coordinate $x=0$) to its distal end ($x=1$).

C = concentration of urea in saliva at point x;
C_0 = concentration of urea in acinar (primary) saliva;
C_1 = concentration of urea in the saliva as delivered to the exterior;
V = rate of saliva water secretion;
C_0' = concentration of urea in the blood water at the acinar end of the periductal capillary;
C_1' = concentration of urea in blood water at the afferent end of the periductal capillary;
V' = rate of flow of blood water in the capillaries. Note that this flow is negative as it is opposite in sense to the flow of the saliva;
k = diffusional constant for the duct.

A parallel equation to Eq. (1) can be set up for radioactive urea in which C_0S_0 is written for C_0, etc. where S with the appropriate modifiers represents the specific activity of the urea at the given point. (By definition as noted earlier $S = C^*/C$ where C^* = radioactive concentration and C = total concentration.)

$$[2] \qquad CS = C_0S_0 - \frac{V'}{V+V'}(C_0S_0 - C_0'S_0')\left[1 - \exp\left\{-\left(\frac{1}{V}+\frac{1}{V'}\right)kx\right\}\right].$$

This second equation is not completely rigorous, because S is not in a steady state. However all S's are in a *proportional steady state* in that

$$\frac{dS}{dt} = a\frac{dS_0}{dt} = b\frac{dS_1}{dt} = c\frac{dS_0'}{dt} = d\frac{dS_1'}{dt},$$

where a, b, c, d are constants. Since all the terms in Eq. [2] have been multiplied by an S the steady state is maintained. A full treatment of the problem is given by Schumann (26).

The over-all transfer of urea across the ducts is given by the conservation equation:

$$[3] \qquad V\frac{dC}{dt} = -V'\frac{dC'}{dt},$$

which, when integrated, gives

$$[4] \qquad C' = C_0' - \frac{V}{V'}(C - C_0).$$

The corresponding equation for radioactive urea is

$$[5] \qquad C'S' = C_0'S_0' - \frac{V}{V'}(CS - C_0S_0).$$

Equations [4] and [5] may be used to eliminate C_0' and S_0' from Eqs. [1] and [2].

By eliminating the factor $\left[1 - \exp\left\{-\left(\frac{1}{V}+\frac{1}{V'}\right)kx\right\}\right]$ between Eqs. [1] and [2] thus modified we obtain

$$[6] \qquad C_0 = \frac{C'C\,(S - S')}{C'S_0 - CS - C'S' - CS_0}.$$

Taking the boundary value $x = 1$ this becomes

$$[6a] \qquad C_0 = \frac{C_1'C_1\,(S_1 - S_1')}{C_1'S_0 - C_1S_1 - C_1'S_1' - C_1S_0}.$$

In the right hand portion of Eq. [6*a*] all the parameters are available from experimental data. (Note: by definition the primary secretion must have the same specific activity as the cells hence $S_0 = S_c$.) In an unloading experiment $S_1' = 0$ and the equation may be further simplified to

$$[6b] \qquad C_0 = \frac{C_1'C_1S_1}{C_1'S_0 + C_1S_1 - C_1S_0}.$$

Finally the complete equation for urea concentration in the ducts may be written explicitly in terms of the diffusional constant k

$$[7] \quad C = \frac{C_1'C_1S_1}{C_1'S_0 + C_1S_1 - C_1S_0} - \left(\frac{C_1'C_1S_1}{C_1'S_0 + C_1S_1 - C_1S_0} - \frac{C_1'V' + C_1V}{V+V'}\right)\left[1 - \exp\left\{-\left(\frac{1}{V}+\frac{1}{V'}\right)kx\right\}\right].$$

TABLE IV

EQUILIBRATION OF UREA IN THE SALIVARY DUCTS

Stimulation frequency	V	V'	C_0	C_1	C_0/C_1	k
0.4	0.041	−0.273	21.7	60.7	0.36	0.030
2	0.041	−0.263	24.0	66.5	0.36	0.037
1	0.090	−0.440	20.9	53.0	0.39	0.050
2.5	0.101	−0.637	23.6	47.4	0.50	0.040
20	0.140	−1.09	7.6	44.8	0.17	0.074
6	0.185	−1.565	11.3	44.0	0.25	0.089
20	0.318	−1.16	20.4	42.0	0.49	0.107
20	0.340	−1.02	7.8	40.0	0.20	0.155
					Mean ± S.E.	0.34 ± 0.044

In Table IV the values obtained by applying these equations to eight experiments with urea are tabulated. The concentration of urea in the primary saliva averaged 34± 4.4% of the concentration in the saliva reaching the exterior, so that approximately two-thirds of the urea in the saliva is added as the saliva passes along the ducts. The amount of urea crossing the duct wall was roughly proportional to the rate of saliva secretion. The duct diffusion constant k also increased with the rate of saliva secretion (Fig. 12). Since the mathematical treatment makes due allowance for the direct effects of increased flow, this means that the condition that leads to increased saliva flow has an effect on the ducts, increasing their permeability to urea.

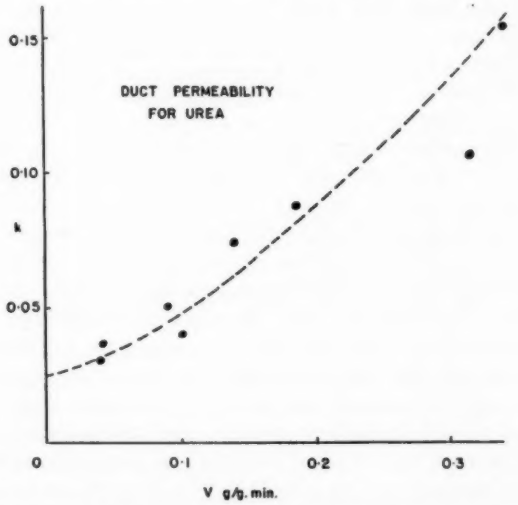

FIG. 12. Relationship between the permeability of the duct for urea (k) calculated from the countercurrent equation 7 and the rate of saliva secretion. Data from 8 experiments. — — — Calculated relationship if the acinar urea permeability is not modified by nerve stimulation.

TABLE V

CORRELATION OF CALCULATED DUCT TRANSFER OF RADIOACTIVE UREA AND THE "EXCESS" VENOUS LOSS RATE, C.P.M./G. MINUTE

Exptl. excess venous loss rate	Calc. transductal loss	Calculated / Experimental
190	231	1.22
182	216	1.19
156	185	1.19
490	404	0.82
320	230	0.72
55	45	0.82
166	156	0.94
	Mean ± S.E.	0.986 ± 0.079

It was noted earlier that during stimulation there was an excess loss rate of C^{14}-urea in the venous blood from the gland (Fig. 5). Clearly some of this labelled urea was secreted into the acini and then diffused across the duct wall into the blood. The correlation between the experimentally determined excess loss rate and the loss across the ducts calculated from the countercurrent equations is very good (Table V). This would only be the case if the permeability of the outer (blood) face of the acinar cells were little affected by stimulation of the gland and hence the rate of loss of radioactive urea directly into the blood were constant. Furthermore, the amount of urea in the primary secretion can be quite well accounted for in terms of a constant permeability of the inner face of the cell. In Fig. 11 the dotted line is the calculated relationship between k and V expected if the permeability of acinar cells were unaffected by nerve stimulation. These considerations make it possible that the permeability to urea of the acinar face of the cell is also unaffected by the secretory process. In a previous paper (16) in which the concentration of nonelectrolytes in saliva was studied as a function of the rate of saliva secretion it was concluded from a simplified kinetic treatment that nonelectrolyte secretion occurred at two sites in the gland. The properties of the first site corresponded to a lipoid membrane of constant permeability and the second to a porous structure whose permeability was dependant on the rate of saliva secretion. The present experiments make it probable that these two sites are the acinar cells and the duct cells respectively.

Extension of the quantitative treatment of countercurrent duct diffusion as applied to urea to the electrolytes of saliva involves very great difficulties. These arise because the mathematics so far developed is only applicable to a substance that diffuses passively along a simple concentration gradient. With electrolytes we immediately encounter the strong probability that at least some of the electrolytes are transferred by "carrier mediated" or active processes whose kinetics differ from the simple Fick kinetics adequate for nonelectrolytes. In addition, the concentration gradient alone is not the unique driving force but electrostatic gradients must also be considered.

These latter impose restrictions on the freedom of individual ion movements because of the need to maintain an over-all electroneutrality.

However, the presence of the potassium transient gives evidence that not only does potassium exchange occur across the ducts, but there is also a net outward movement of potassium across the duct wall. Also, the very low specific activity of the sodium in the saliva during an unloading experiment can best be accounted for as mainly due to isotopic dilution of the salivary sodium by a net inward movement of sodium from the blood along the over-all concentration gradient. The concentration of sodium in the saliva is a positive function of the rate of secretion of saliva (5, 27, 28) as are the concentrations of bicarbonate and chloride. At low rates of saliva secretion the sodium concentration may be only 1–2 meq./l. and unless active reabsorption of sodium is occurring in the ducts, this would in turn mean that the sodium concentration in the primary secretion may be very low.

The bicarbonate of the saliva is especially interesting in that it may reach approximately twice the plasma concentration (5, 6). Sand (29) has shown that the bicarbonate of saliva is of mixed origin, being derived in part from the plasma bicarbonate but also to a considerable extent from metabolic carbon dioxide produced in the gland. These results suggest that a major part of the bicarbonate is probably secreted by the acini and that little, if any, is added by secretory activity of the tubule cells.

The sum of our deductions about the four major ions is that the primary secretion is rich in potassium and bicarbonate and probably poor in sodium. The chloride level may be intermediate. Unfortunately we have no information that can help us in deciding the total osmolarity of the primary secretion, but on the basis of a primary secretion isotonic with the cell contents and with the ionic proportions postulated, a scheme of the secretory process can be drawn up which provides a rational basis for the many unusual features discovered in the gland secretory process.

The process might be somewhat as follows. The acinar cells secrete potassium ions and bicarbonate ions into the acinar lumen — enough chloride accompanies them to maintain electroneutrality. Because both cations and anions are secreted in step, little or no change in potential occurs across the inner cell face (30, 31). If the inner cell face is freely permeable to water, water movement will accompany the ion transfer to maintain the primary secretion isotonic with the cell contents. This transfer of ions will involve little work as hardly any concentration change is being produced. In the ducts potassium leaves the saliva and carries with it an anion. At the same time some sodium enters the saliva from the blood. The loss of potassium and an associated anion with only partial replacement by sodium leads to the characteristic hypotonicity of the saliva. The final equilibrium is due to the high diffusibility of potassium and the relatively low diffusibility of sodium and bicarbonate. For a hypotonicity to be produced the duct cells must be relatively impermeable to water molecules, but this must be assumed whatever secretory scheme is proposed. Precedents also exist. For instance, red blood cells of the ox are about five times as permeable to urea as to water (32).

The potassium transferred across the duct wall is picked up by the duct countercurrent capillary system and carried to the acini where it is reaccumulated by the cells and made available for secretion again. The duct countercurrent system can be regarded as an economizer of potassium in supplying the acini with potassium at a higher concentration than in the arterial plasma. The potassium transient in the venous blood is an indicator of this potassium cycle. At the beginning of stimulation the acinar cells are not depleted of potassium and hence their ability to pick up potassium from the blood is not very great, and some of the potassium reabsorbed by the ducts escapes into the venous blood. As the cells become depleted of potassium their avidity for the plasma potassium is increased and a steady state is set up. It is probable that there is an increased rate of secretion of potassium at the beginning of stimulation and that a transiently high rate of saliva secretion accompanies this phase (10). The major work of saliva secretion in this scheme is the work of accumulating potassium in the cells from the plasma potassium. The concentration work done is given by

$$W = nRT \ln c'/c.$$

If at the maximum rate of secretion of 0.3 ml./g. minute, the concentration of potassium in the primary saliva is 140 meq./l., and the concentration in the donor plasma is 4 meq./l., then the concentration work would be 0.10 cal./g. minute. Barcroft (33) found that 0.5 cal./g. minute was available from respiration. The energy demands of potassium concentration in this system are therefore not excessive.

The scheme of secretion proposed here has the virtue of providing a rational explanation of the ionic composition of the saliva, the countercurrent duct portal system, the venous potassium transient, and the formation of hypotonic saliva. It is fully realized that it is merely a working hypothesis on which to base further investigation and cannot be regarded as in any way established. If it can be firmly established it may provide a useful model for other secreting glands in which the evidence is suggestive that the potassium ion is somehow concerned with secretion. It may be remarked here that the sheep parotid gland shows a potassium transient analogous to that in the dog's gland (34). This gland normally secretes a nearly isotonic saliva in which the major cation is sodium, although the potassium concentration in the saliva is higher than in the plasma. When the sheep is depleted of sodium these ions change places and the parotid saliva can become very rich in potassium (up to 130 meq./l.) and poor in sodium (35, 36). This change is prevented by adrenalectomy. A similar though smaller change in sodium and potassium content of saliva is produced in man by adrenalcorticoids (37,38). These results are interesting because they suggest that at any rate in the salt-depleted sheep the saliva may have the composition we have postulated for the primary secretion. The effects of the adrenal corticoids might be due to a diminished permeability of the ducts to both sodium and potassium, thus keeping the composition of the secreted saliva to a closer replica of the primary concentration.

The difference of composition of sheep parotid saliva from that in man, dog, or cat can be explained on the supposition that the ducts of this species are much more permeable to sodium than in non-ruminants so that the limiting permeability in this case is to potassium. A similar explanation may apply to the sublingual gland in man and cat (30, 33).

However, some modification of this scheme is made necessary by the work of Lundberg (30, 31, 40) on the membrane potentials of salivary gland cells. Lundberg found that an intracellular electrode in a gland cell was about 30 mv. negative to the exterior across both blood and acinar faces of the cells. There seems little reason to doubt that this is an example of a genuinely low cell membrane potential. The potential does not agree with the Nernst equation for potassium ($K_e/K_0 \backsimeq 35$; $E_K = -95$ mv.) but on the other hand the internal chloride of the salivary cells is about 25 meq./kg. H_2O (Burgen, unpublished), which gives a chloride ratio of $Cl_e/Cl_0 \backsimeq 0.25$ and a chloride potential of $E_{Cl} = -36$ mv. in reasonable agreement with the measured potential. This could mean either that chloride is passively distributed, or that the membrane potential of the salivary cells is chloride dominated. Lundberg also found that stimulation of the parasympathetic nerves to the gland produced a striking hyperpolarization of the outer cell face averaging about 30 mv. This hyperpolarization is unique in that it is independent of the resting potential of the cell and thus differs from the synaptic hyperpolarization occurring in anterior horn cells, crustacean stretch receptors, and the heart (41), in all of which an equilibrium membrane potential can be found at which the presynaptic volley produces no membrane potential change. Lundberg concluded that this hyperpolarization must be due to an active ion movement and since in general the salivary gland is transferring ions in the direction of extracellular fluid to secretion, active *anion* transfer into the cell is required to account for the sign of the potential change. In the perfused sublingual gland, substitution of nitrate for chloride (40) virtually abolishes both the secretion and the hyperpolarization of the cell that normally result from the application of acetylcholine. It is therefore possible that chloride is the ion involved in generating the hyperpolarization. A further point to be taken into account is that during the hyperpolarization the conductance of the outer cell face increases by about 60%. Now we can assume that the membrane conductance is entirely due to Na, K, and Cl ions in that the contribution of other ions is almost certainly trivial. We have shown both Na and K fluxes increase at least 10-fold during secretion.

Therefore if at rest

$$G_{Na+K} + G_{Cl} = 1.00$$

(where G_{Na+K} = fractional resting conductance due Na+K ions;

G_{Cl} = fractional resting conductance due Cl ions),

then during activity the conductances can be represented by

$$10\ G_{Na+K} + xG_{Cl} = 1.60,$$

where x is the ratio of active to resting chloride conductance and is defined by the limits $1 < x < 1.6$.

Since a considerable extra transfer of chloride ions is needed to generate the secretory hyperpolarization, x is probably nearer to 1.6 than 1. An intermediate value of 1.3 will serve for illustration and leads to the conclusion that the cation fluxes are responsible for only a few per cent of total membrane conductance and that the chloride flux is very large (of the order of 50 μeq./g. minute). This conclusion fits well with the idea that the resting potential in the acinar cells is chloride dominated. Unfortunately the probable magnitude of this chloride flux excludes direct measurement with present methods. This evidence that active anion transport is important in the salivary gland leads to the proposition that the increased potassium entry into the gland during activity may be in part an electrostatic concomitant of the hyperpolarization and increased anion movement.

The apparent dominance of chloride ions in the electrochemical processes of the salivary gland may be compared with its importance in gastric secretion (42, 43), and indeed the two organs appear to share other features of their secretory processes.

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THE HYDROLYSIS OF *p*-ACETOXYPHENYLETHYLAMINES BY INSECT CHOLINESTERASE[1]

L. S. WOLFE AND G. D. THORN

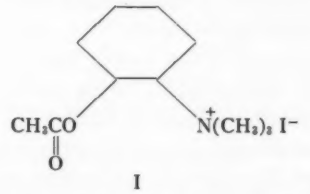
Abstract

The synthesis of the acetyl derivatives of tyramine and hordenine is described. The O-monoacetyl derivatives are hydrolyzed at significant rates by bovine erythrocyte cholinesterase, human serum, and fly head cholinesterase despite a nitrogen to carbonyl oxygen separation approximately twice that of acetylcholine. The pS-activity relationships, when O-acetyltyramine and acetylcholine were substrates for fly head cholinesterase, were similar, but the hydrolysis rate of O-acetyltyramine was much higher than that of acetylcholine. N-Acetylation of the O-acetyl compounds reduced the hydrolysis rate. None of the cholinesterases removed the acetyl group attached to nitrogen. The pI-activity relationships with the inhibitors Nu-683, Nu-1250, TEPP, and eserine showed that the hydrolysis of *p*-acetoxyphenylethylamine derivatives and acetylcholine by fly head preparations was accomplished by the same cholinesterase and not by aromatic or aliesterases. O-Acetylation of hordenine methiodide destroyed its nicotinelike action on frog rectus abdominis muscle.

Introduction

Mounter and Whittaker (13, 14) showed that "true" cholinesterases were capable of hydrolyzing esters other than those of choline. Nachmansohn and Wilson (see 15) demonstrated the existence of two active sites for each cholinesterase center, one anionic and the other esteratic. The substrate molecule combines with the enzyme at both these sites before hydrolysis. Baldridge, McCarville, and Friess (2), and Friess and McCarville (9), studied the structure of competitive inhibitors of cholinesterase and the required separation of the anionic and cationic sites on the enzyme surface necessary to give optimal substrate or inhibitor activity. They reported that the *cis* and *trans* isomers of 2-acetoxy-cyclohexyl-trimethyl ammonium iodide (I and II) acted as substrates for the cholinesterase from the electric eel.

In this paper, the hydrolysis of acetylated *p*-hydroxyphenylethylamines by cholinesterase preparations is studied. These compounds are O-acetyltyramine hydrochloride (*p*-acetoxyphenylethyl ammonium chloride) (III), O-acetylhordenine methiodide (*p*-acetoxyphenylethyl trimethyl ammonium iodide) (IV), O-acetylhordenine hydriodide, N-acetyltyramine, O,N-diacetyltyramine, and O,N,N-triacetyltyramine. In neutral solution, the first three of these compounds possess an ionized nitrogen, the latter three being incapable of salt formation because of the acetyl group on the nitrogen.



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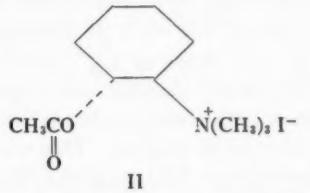

II

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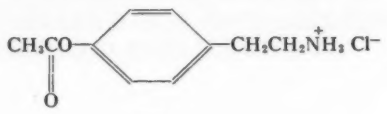
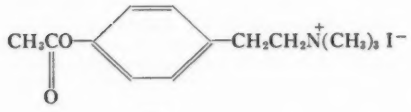


III



IV

Preparation of Materials

Of the five possible acetyl derivatives of tyramine only the preparation of the O,N-diacetyl compound (7) was found in the chemical literature. In our hands the method of Cloetta and Wünsche (7) yielded only O,N,N-triacetyltyramine. This compound was obtained also by following the procedure of Buck and Baltzly (5) which these authors used for the O-acetylation of N-methyltyramine. The O,N-diacetyl compound was obtained with Chattaway's method (6) provided that the pH of the reaction medium did not exceed 8.0. A final pH above 8.0 resulted in the formation of N-acetyltyramine (see Sealock (17) on the acetylation of tyrosine). The N-acetyl derivative was obtained also by reacting tyramine with isopropenyl acetate (8) in diethyleneglycol dimethyl ether without an acid catalyst. The acetylation method of Bretschneider (3) and Bretschneider and Biemann (4) yielded the O-acetyltyramine hydrochloride. Details of the preparation of the acetyl derivatives of tyramine and hordenine are given below.

O,N,N-Triacetyltyramine

A. A solution of 0.60 g. tyramine and 0.25 g. anhydrous sodium acetate in 5.0 ml. acetic anhydride was heated under reflux for 3 hours. Water was added, and the mixture extracted with ether. Removal of the solvent from the ether layer and crystallization of the residue from ethyl acetate and then from ethanol gave 0.35 g. (36%) of a white material, melting at 90–91°. Found: C, 63.7; H, 6.71; N, 5.33%. Calc. for triacetyltyramine, $C_{14}H_{17}O_4N$: C, 63.9; H, 6.47; N, 5.33%. The infrared absorption spectrum of this material displayed two carbonyl peaks, 1755 cm.$^{-1}$ (O-acetyl) and 1685 cm.$^{-1}$ (N-acetyl) and no N–H absorption in the 3300 cm.$^{-1}$ region.

B. A mixture of 1 g. tyramine hydrochloride, 2 ml. acetyl chloride, and 8 ml. freshly distilled acetic anhydride was heated under reflux for 4 hours. The volatile components were then removed with a stream of nitrogen to leave a tan colored residue. This was twice crystallized from 1,2-dimethoxyethane (Ansul ether 121) to give 0.65 g. (43%) of the triacetyl derivative, melting at 90–91°. A mixture of this material with that from A above melted at 90–91°. Found: C, 63.9; H, 6.50%. Calc. for $C_{14}H_{17}O_4N$: C, 63.9; H, 6.47%.

O,N-Diacetyltyramine

Tyramine hydrochloride, 1.73 g., was dissolved in 5 ml. water and 10 g. ice containing 1.20 g. sodium hydroxide. Acetic anhydride, 2.0 ml., was added quickly and the mixture shaken. The pH was raised to 8.0 with 10% sodium hydroxide and the shaking continued for 5 minutes. The white precipitate which formed was removed by filtration, washed with water, and dried *in vacuo* over sodium hydroxide. The yield of crude material was 1.06 g. (48%), melting 91–94°. Repeated crystallization from ether–dimethoxyethane resulted in fine long needles with a constant melting point of 100–101° (Literature (7), 103°.). Found: C, 65.3; H, 6.76; N, 6.32%. Calc. for $C_{12}H_{15}O_3N$: C, 65.2; H, 6.79; N, 6.34%.

The infrared spectrum showed two carbonyl peaks, 1760 cm.$^{-1}$ (O-acetyl) and 1642 cm.$^{-1}$ (N-acetyl) and N–H absorption at 3290 cm.$^{-1}$

N-Monoacetyltyramine

Tyramine, 2.74 g., was added to 2.00 g. isopropenyl acetate in 5 ml. diethyleneglycol dimethyl ether (Ansul ether 141) and the mixture heated under reflux for 4 hours. The acetone and any unreacted isopropenyl acetate were removed by distillation, and 10 ml. hexane was added to the residue. The crystals which formed on cooling were removed by filtration and crystallized twice from ethyl acetate. The yield of white blunt needles, melting at 132.5–133.5°, was 2.40 g. (67%). Found: C, 67.2; H, 7.70; N, 7.86%. Calc. for $C_{10}H_{13}O_2N$: C, 67.1; H, 7.27; N, 7.83%. The infrared spectrum showed only one carbonyl peak, at 1635 cm.$^{-1}$ (N-acetyl), and possessed both N–H and O–H absorption.

O-Monoacetyltyramine Hydrochloride

Tyramine hydrochloride, 1.00 g., was added to 4 ml. glacial acetic acid saturated with hydrogen chloride and 3 ml. acetyl chloride. After the mixture had stood overnight, a further 10 ml. of HCl-saturated acetic acid and 4 ml. of acetyl chloride were added, the whole was warmed to 80° for a few minutes (tightly stoppered flask) and then allowed to stand for 2 hours. The volatile components were removed in a stream of nitrogen, and the residue was dried *in vacuo* over phosphorous pentoxide and sodium hydroxide pellets. Recrystallization was best achieved by solution of the material in a minimum of absolute methanol at room temperature, followed by the addition of sufficient ethyl acetate to cause cloudiness. Chilling resulted in the formation of shining white plates, weighing 0.85 g. (82%), and melting at 167–168°. Found: C, 55.5; H, 6.46; N, 6.55%. Calc. for $C_{10}H_{14}O_2NCl$: C, 55.8; H, 6.98; N, 6.52%. The infrared absorption spectrum of this compound shows but one carbonyl band, at 1750 cm.$^{-1}$ (O-acetyl).

O-Acetylhordenine Hydriodide

This compound was prepared according to the method of Späth (18). Hordenine, 0.73 g., and 7.5 ml. acetic anhydride were heated under reflux for ½ hour, then allowed to stand overnight. The acetic acid and anhydride

were removed with nitrogen, to leave a colorless oil. Four-tenths of this oil was dissolved in a few milliliters of water, and to this solution were added 0.5 ml. 3 *N* HCl and 0.5 g. potassium iodide. The white plates which formed were removed by filtration. The dry yield was 0.40 g. (71%). The material melted at 176–177°. (Literature records the melting point as 175–176° (18).) Found: I, 38.1%. Calc. for acetylhordenine hydriodide, $C_{12}H_{18}O_2NI$: I, 37.9%.

O-Acetylhordenine Methiodide

The remaining 6/10ths of the oil from the acetylation of hordenine, above, was dissolved in a little absolute methanol, and 0.5 g. methyl iodide added. Upon standing, 0.80 g. (83%) of a white material separated out. The melting point was 258–260°. Found: C, 44.8; H, 6.00%. Calc. for acetylhordenine methiodide, $C_{13}H_{20}O_2NI$: C, 44.7; H, 5.73%.

Hordenine Methiodide

This compound was prepared according to Späth (18) in almost quantitative yield. The melting point was 232.5–233.5°. (Lit. records 231° (18).) Found: I, 41.1%. Calc. for $C_{11}H_{18}ONI$: I, 41.4%.

Methods

Enzyme Preparations

Purified bovine erythrocyte cholinesterase, General Biochemicals, 12.5 mg./25 ml. Diluted human serum. Housefly heads homogenized in a Potter–Elvehjem homogenizer in distilled water, 10 heads/ml.

Substrates

Tyramine and hordenine esters, the preparation of which is described above. Acetylcholine bromide. The solutions were made up freshly each day in 0.025 *M* $NaHCO_3$ solution.

Inhibitors

The prostigmine analogues Nu-683 (the dimethylcarbamate of 2-hydroxy-5-phenylbenzyl trimethyl ammonium bromide) and Nu-1250 (the N-*p*-chlorophenyl-N-methyl carbamate of *m*-hydroxy-phenyl trimethyl ammonium bromide) were obtained from Hoffmann-LaRoche, Basel, Switzerland (see Hawkins and Gunter (10) and Hawkins and Mendel (11)). TEPP (tetraethyl pyrophosphate) was synthesized by Dr. J. R. Robinson of this laboratory.

Eserine Sulphate

Stock solutions were prepared on the day of the experiment in distilled water and final concentrations obtained by serial dilutions.

Measurement of Enzyme Activity

Cholinesterase activity was determined by the manometric method. The conditions of assay were: 0.5 ml. enzyme preparation; 2.0 ml. $NaHCO_3$ (0.025 *M*, final); 0.2 ml. substrate (0.01 or 0.005 *M*, final); final pH was 7.4, temperature 25°. All determinations were made in triplicate and corrected

for spontaneous hydrolysis of the substrates, and activities were calculated as b_{30}'s by the method of Aldridge, Berry, and Davies (1). Inhibitors were incubated with the enzyme–bicarbonate mixture in the main compartment of the flasks for 1 hour before addition of the substrate.

Results

In Table I the hydrolysis rates of the acetylated tyramine and hordenine compounds by bovine erythrocyte cholinesterase, human serum, and fly head cholinesterase, respectively, are compared to the hydrolysis rate with acetylcholine as substrate. O-Acetyltyramine hydrochloride was hydrolyzed at slower rates than acetylcholine by both mammalian preparations. The housefly head enzyme hydrolyzed the O-acetyltyramine at a considerably faster rate than acetylcholine. Although all the O-acetylated compounds were hydrolyzed faster by fly head enzyme than by the mammalian enzymes, it is doubtful whether any significance can be attached to a vertical comparison of the results shown in Table I, as it cannot be assumed that the turnover rates of the enzyme in the three preparations are the same. The hydrolysis rates for O,N,N-triacetyltyramine as substrate were 1–2% of that for acetylcholine with all three enzyme preparations, and are not included in Table I as the determinations were made at 0.005 *M* substrate because of low water solubility. None of the cholinesterases would remove acetyl attached to nitrogen. The nitrogen in the N-acetylated tyramines does not have the ionic character of the nitrogen in O-acetyltyramine and O-acetylhordenine. Hence, the N-acetylated compounds might not be attached to the anionic site of a cholinesterase center. It is thought that the lower hydrolysis rates of the O,N-acetylated compounds are related to this lack of ionic character.

The pS plots for the hydrolysis of O-acetyltyramine and acetylcholine by housefly head cholinesterase are shown in Fig. 1. The activity–concentration relationships are strikingly similar, with maximal hydrolysis occurring at the same substrate concentration (0.01 *M*).

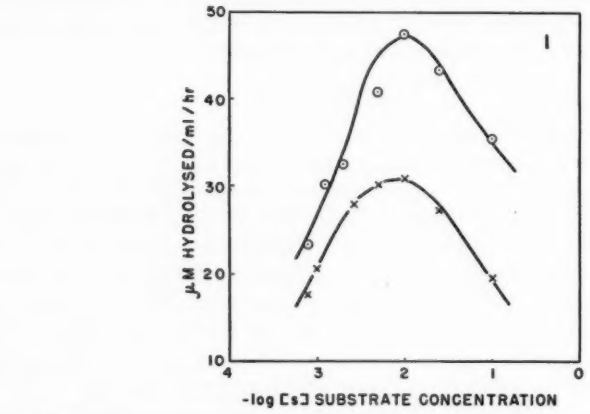
To demonstrate clearly that the fly head acetylcholinesterase was responsible for the hydrolysis of both substrates rather than non-specific esterases or aliesterases the effects of the inhibitors Nu-683 and Nu-1250, and TEPP

TABLE I

COMPARISON OF HYDROLYSIS RATES OF ACETYLTYRAMINES AND ACETYLHORDENINES BY VARIOUS CHOLINESTERASE PREPARATIONS, EXPRESSED AS % OF THE ACETYLCHOLINE RATE

pH 7.4; substrate concentration, 0.01 *M*; temp. 25°

ChE source	O-Acetyl-tyramine	O,N-Diacetyl-tyramine	N-Acetyl-tyramine	O-Acetyl-hordenine hydriodide	O-Acetyl-hordenine methiodide
Purified bovine erythrocyte	40.2	12.4	0	10.1	14.5
Human serum	78.3	38.1	0	35.7	44.4
Housefly head	131.8	76.4	0	73.5	77.0



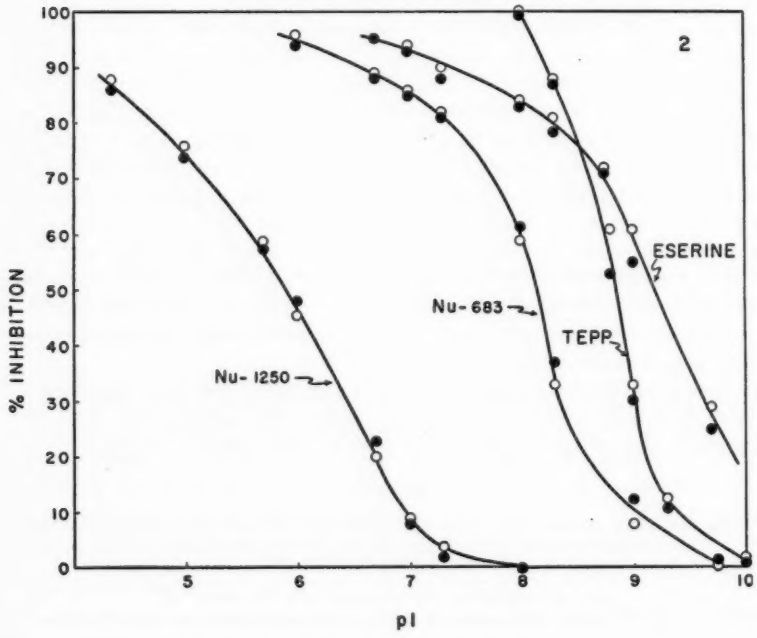


FIG. 1. Comparison of the hydrolysis of O-acetyltyramine hydrochloride (—○—) with acetylcholine bromide (—×—) by housefly head cholinesterase at various substrate concentrations.

FIG. 2. Inhibition of housefly head cholinesterase by Nu-683, Nu-1250, TEPP, and eserine sulphate. —○—, Acetylcholine bromide as substrate. —●—, O-Acetyltyramine hydrochloride as substrate.

and eserine sulphate were studied. The pI plots with O-acetyltyramine and acetylcholine as substrates are shown in Fig. 2. It is evident that for each inhibitor almost identical plots were obtained for the inhibition of the hydrolysis of the two acetyl compounds. If O-acetyltyramine was being hydrolyzed by esterases other than acetylcholinesterase this would not be so. The pI-50 values were: Nu-1250, 5.9; Nu-683, 8.1; TEPP, 8.9; eserine sulphate, 9.5.

Hordenine methiodide will induce contraction of the frog rectus abdominis muscle, resembling the action of nicotine and of acetylcholine. O-Acetylhordenine methiodide (e. g., 2×10^{-8} *M*) was without effect on a frog rectus abdominis muscle preparation, but after the addition of a small quantity of fly head cholinesterase to the muscle bath, a strong contraction of the muscle was produced after a brief delay. This is contrary to the case of choline which is far less potent in producing contraction than its acetylated derivative. O-Acetylhordenine hydriodide, hordenine hydriodide, O-acetyltyramine hydrochloride, and tyramine did not cause contraction of the frog muscle.

Discussion

The purpose for preparing the acetyl derivatives of tyramine and hordenine was to investigate alternative substrates for insect cholinesterase which possess an ionized nitrogen separated from a carbonyl group as occurs in acetylcholine but with different N → O distances. It was found that purified bovine erythrocyte cholinesterase, crude human serum, and fly head cholinesterase were capable of hydrolyzing at significant rates *p*-acetoxyphenylethyl ammonium compounds. Clearly, therefore, the distance between the quaternary nitrogen and the acetoxy groups is not the *sine qua non* for ability to be a substrate for cholinesterase.

O'Brien (16) has shown that fly head cholinesterase differs from erythrocyte cholinesterase in its behavior with certain organophosphate inhibitors. Further evidence for this is given by the hydrolysis rates of O-acetyltyramine and acetylcholine. In fly heads all the cholinesterase is present in nervous tissue and Wolfe and Smallman (19) showed that it is almost entirely acetylcholinesterase (specific cholinesterase). Metcalf *et al.* (12) have shown that in insect nervous tissue there is also a small amount of an aromatic esterase and aliesterase. The results reported here indicate that the specific cholinesterase of fly heads also hydrolyzes certain aromatic esters. The close similarity of the pS-activity relationships and the identical pI-activity plots obtained when acetylcholine and O-acetyltyramine were used as substrates must mean that the enzyme hydrolyzing both substrates was the same enzyme and was an 'acetylcholinesterase' and not an aliesterase or aromatic esterase.

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PREPARATION AND MOLECULAR WEIGHT OF γ-LIVETIN FROM EGG YOLK[1]

W. G. Martin and W. H. Cook

Abstract

A 20% solution of egg yolk in ammonium sulphate at 16% of saturation was centrifuged to float the low density components and the γ-livetin was then precipitated from the subnatant with ammonium sulphate at 40% of saturation. The precipitate was dissolved and reprecipitated several times with ammonium sulphate at 34% of saturation before ether extraction. γ-Livetin prepared in this way contained only a small amount of a heavier component. On purification γ-livetin has the solubility behavior of a euglobulin but loses its solubility irreversibly on prolonged dialysis against water or on freeze-drying. It can be preserved for reasonable periods as a precipitate in saturated ammonium sulphate solution. γ-Livetin has an anomalous sedimentation behavior, the sedimentation rate increasing sharply over a short range of protein concentration. A molecular weight of 1.5×10^6 and an axial ratio of about 8 were obtained from sedimentation and diffusion measurements. Other methods gave higher values but are considered less reliable.

Introduction

The globulin-like protein in the egg yolk of the domestic hen has been termed livetin (5, 9) and electrophoretic analyses have shown it to contain three major components (8, 11, 12). The separation of these components, termed α-, β-, and γ-livetin in order of decreasing electrophoretic mobility, was described in a paper from these laboratories (8). The molecular weights of α- and β-livetin were estimated to be 4.2 and 8.0×10^4 respectively. The sedimentation coefficient of γ-livetin indicated that its molecular weight was greater than that of the other livetins but the instability of this fraction did not permit adequate physical measurements to be made at that time. This paper reports additional information on the preparation, properties, and molecular weight of γ-livetin.

Experimental

Methods

Chemical and physical methods were essentially as described in the previous paper (8). Hexose and hexosamine were determined by the methods described by Winzler (17). A MacInnes float apparatus (1, 7) was used to obtain the partial specific volume ($\bar{v}$) at 25° C. Viscosity determinations were made in an Ostwald–Fenske type viscometer with a flow time of 290 seconds. Diffusion coefficients were determined from measurements made in an integral fringe diffusion apparatus constructed from Hilger schlieren optical equipment modified to give a Rayleigh system of the type used by Longsworth (6).

Solutions used for light-scattering determinations were centrifuged for 1 hour in a Spinco preparative centrifuge at 30,000 r.p.m., drawn into a dust-free

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pipette, and filtered through millipore filters into the light-scattering cell which contained previously-filtered solvent. The cell was weighed at each addition and the concentrations calculated from the final solution concentration in the cell as determined in a Beckmann spectrophotometer.

Preparation

Initial attempts to prepare γ-livetin were based on the older procedures for preparing livetin. Lipovitellin was precipitated by dilution and the supernatant treated with ethyl ether to extract the "free" lipid and facilitate removal of the residual lipovitellenin that collects at the interface. The γ-livetin was then precipitated from the aqueous layer by the addition of ammonium sulphate to 37% of saturation (8). By this procedure the γ-livetin often became insoluble before the other proteins were removed, and at best resulted in extremely low yields of γ-livetin of uncertain stability.

The initial attempts at preparation indicated that: γ-livetin and lipovitellin were difficult to separate, since they have similar properties and may indeed interact; γ-livetin is unstable and loses solubility readily during preparation. The proportion of lipovitellin was reduced by precipitating the γ-livetin at 34% of saturation with ammonium sulphate although this also reduced the yield. Differential precipitation of the γ-livetin was less effective in the presence of ether, and to avoid the steps necessary for its removal, the γ-livetin was purified before ether extraction.

Exhaustive dialysis against water precipitated γ-livetin, but within reasonable periods it could be redissolved in solvents of low ionic strength ($\mu = 0.1$). This indicated that purified γ-livetin had the solubility behavior of a euglobulin rather than a pseudoglobulin (5). If the precipitate obtained on dialysis against water was allowed to stand, it gradually lost its solubility in dilute salt solutions. In some buffers γ-livetin would gradually precipitate, and even clear solutions occasionally contained aggregates that formed a gel-like sediment on centrifuging.

The solubility of γ-livetin was examined in several solvent systems. Precipitation occurred in acetate buffer at pH 4.6, and in glycine–HCl buffer at pH 3.1, both at 0.2 ionic strength. Slow precipitation occurred in sodium chloride solutions at $\mu = 0.2$. Solutions in glycine or veronal buffers at $\mu = 0.2$, at pH > 8, remained clear for a week at 5° C. and these buffers were used for all subsequent physical examinations.

The precipitate that formed in a solution of γ-livetin in 0.2μ sodium chloride was insoluble in 2-mercaptoethanol, dimethyl sulphoxide, and glacial acetic acid. It was soluble in formamide and 90% formic acid.

The following procedure was therefore developed for the preparation and examination of γ-livetin. A dispersion of 20% egg yolk was made in an ammonium sulphate solution at 16% of saturation to provide a solvent of the required density. This was centrifuged 16 hours at 21,000 r.p.m. in a Spinco preparative centrifuge. The clear subnatant solution was removed from the floating low density material by piercing the bottom of the centrifuge

tube and was brought to 40% saturation with ammonium sulphate to precipitate the γ-livetin. This first precipitate contained some lipovitellin and α- and β-livetin. Purification was accomplished by successive solution and reprecipitation from 34% saturated ammonium sulphate solution. This procedure gave a product with the electrophoretic mobility and sedimentation properties of γ-livetin but analyses showed that it contained about 8% lipid. This was removed by ether extraction and on removal of the ether the γ-livetin remained unchanged for several weeks when preserved as a precipitate in saturated ammonium sulphate solution at 4° C. Glycine and veronal buffers of 0.1 and 0.2 ionic strength at pH 8.5 and 9.5 were used as solvents for most of the physical measurements.

Results

The electrophoretic and sedimentation diagrams of one of the preparations of γ-livetin appear in Fig. 1. The minor component in the sedimentation diagram of Fig. 1C represents about 4% of the total protein present and had a sedimentation rate of 12S (Svedberg unit) which is similar to that of lipovitellin under comparable conditions. The electrophoretic diagram shows no evidence of more mobile components such as α- and β-livetin. The γ-livetin peak, however, shows considerable spreading, indicative of the heterogeneity common to most globulins (4, 15). The minor heavy component noted above may also have contributed to the spreading.

A sample of γ-livetin, which had precipitated from a solution of sodium chloride, was dissolved in 90% formic acid and examined in the ultracentrifuge (Fig. 1D). A minor fast sedimenting component equivalent to about 3% of the main peak is also evident in this solvent.

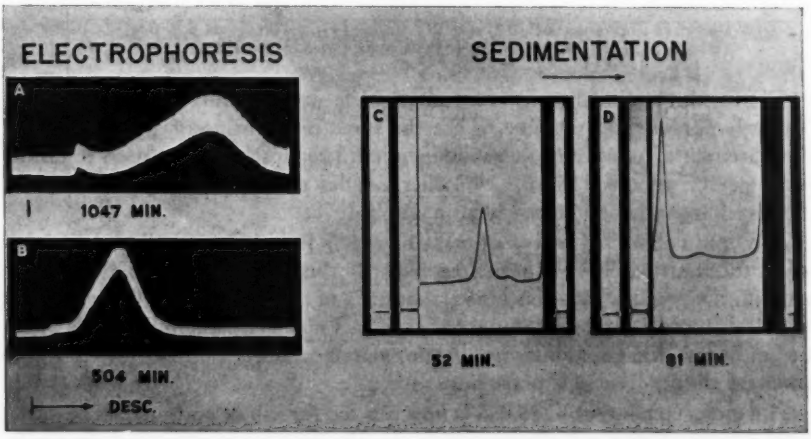

FIG. 1. Electrophoretic (A, B) and sedimentation (C, D) patterns of γ-livetin prepared as described. A and B. In glycine buffer, 0.1 μ, pH 9.5. C. In glycine buffer 0.2 μ, pH 9.5. D. Degraded material in 90% formic acid.

TABLE I

SUMMARY OF CHEMICAL AND PHYSICAL MEASUREMENTS

Property	Value	Remarks
Lipid, %	0.54	Following extraction with ethyl ether
Phosphorus, %	0.03	
Nitrogen, %	15.6	
Hexose, %	2.6	
Hexosamine, %	1.8	
Ash, %	0.6	
Molar tyrosine/tryptophan ratio	1.6	
$dn/dc \times 10^3$, g./dl.		
at $\lambda = 5890$ Å	1.880	at 25° C.
at $\lambda = 4360$ Å	1.94	at 25° C.
$E_0^{1\%}$ at $\lambda = 2800$ Å	13.5	
Mobility $\times 10^5$, cm.^{2}sec.$^{-1}$volt^{-1}	−2.7	Buffer (*a*) see below
$[\eta] \times 10^2$, dl./g.	6.54 ± 0.16	Buffer (*d*) $d\eta_{red}/dc = 1.5 \times 10^{-2}$ at 25° C.
$\bar{v}$, ml./g.	0.726 ± 0.001	Buffers (*a*) and (*d*) gave same value
$S^0_{20,w}$, Svedbergs		
Buffers (*a*) and (*b*)	7.62 ± 0.02	$ds/dc = -1.03$
Buffers (*c*) and (*d*)	7.46 ± 0.01	$ds/dc = -0.18$
Sodium chloride, $\mu = 0.1$	7.61 ± 0.04	$ds/dc = -0.18$
$D_{20,w} \times 10^7$, cm.^{2}sec.$^{-1}$		
Concn. = 0.20% in buffer (*a*)	4.47 ± 0.03	Measured at 20.0° C.
Concn. = 0.30% in buffer (*d*)	4.43 ± 0.02	Measured at 25.0° C.
Concn. = 0.85% in buffer (*d*)	4.66 ± 0.20	Measured at 25.0° C.
$M \times 10^5$		
from *s* and *D*, buffers (*a*) and (*b*)	1.51 ± 0.01	
from *s* and *D*, buffers (*c*) and (*d*)	1.49 ± 0.01	
from light-scattering	2.2 ± 0.07	

NOTE: Buffer (*a*): glycine, pH 9.5, $\mu = 0.1$; buffer (*b*): veronal, pH 8.5, $\mu = 0.1$; buffer (*c*): glycine, pH 9.5, $\mu = 0.2$; buffer (*d*): veronal, pH 8.5, $\mu = 0.2$.

The results of the chemical and physical measurements are summarized in Table I. It was not possible to use the same preparation of γ-livetin for all measurements, but two or more independent preparations were used to obtain most of the reported results. Earlier results on less satisfactory material clustered around the values given in Table I.

The lipid content of γ-livetin was about 8% but this was reduced to 0.5% when the solutions were ether-extracted. If this residual lipid is due to lipovitellin, the preparations contained about 3% of this lipoprotein, in agreement with the amount of minor component observed ultracentrifugally. This quantity of lipovitellin could also account for the phosphorus content (3, 16) if it is assumed that γ-livetin is phosphorus free.

The nitrogen content of 15.6% is uncorrected for ash or non-protein material since it represents the entity on which the physical measurements were made. Although some of the non-protein materials contained nitrogen, the nitrogen content of the protein moiety proper is obviously somewhat higher than the

reported value. The molar tyrosine/tryptophan ratio of 1.6 is lower than the ratio previously reported for α- and β-livetin (8).

The electrophoretic mobility of purified γ-livetin was minus 2.7×10^{-5} cm.²/second/volt. The value previously reported (8) at pH 8.0 in a glycine buffer was minus 2.1×10^{-5} cm.²/second/volt. Comparable low mobilities were observed in other buffers but the combined effect of low mobility and considerable spreading made accurate mobility measurements difficult.

Glycine and veronal buffers of two ionic strengths were used for most of the physical measurements, as indicated in the footnote to Table I. Differences due to these solvents were detectable only by sedimentation methods. The low intrinsic viscosity and generally low concentration dependence in all measurements indicate an essentially spherical molecule. The partial specific volume measured in both buffers over a range of protein concentrations varied only in the fourth place and such differences were beyond the accuracy with which the concentration could be determined.

The sedimentation behavior of γ-livetin is shown in Fig. 2. The most notable point is the increase of about 0.5 S that occurs at protein concentrations between 0.5 and 1% (Fig. 2D). This increase was even sharper in any

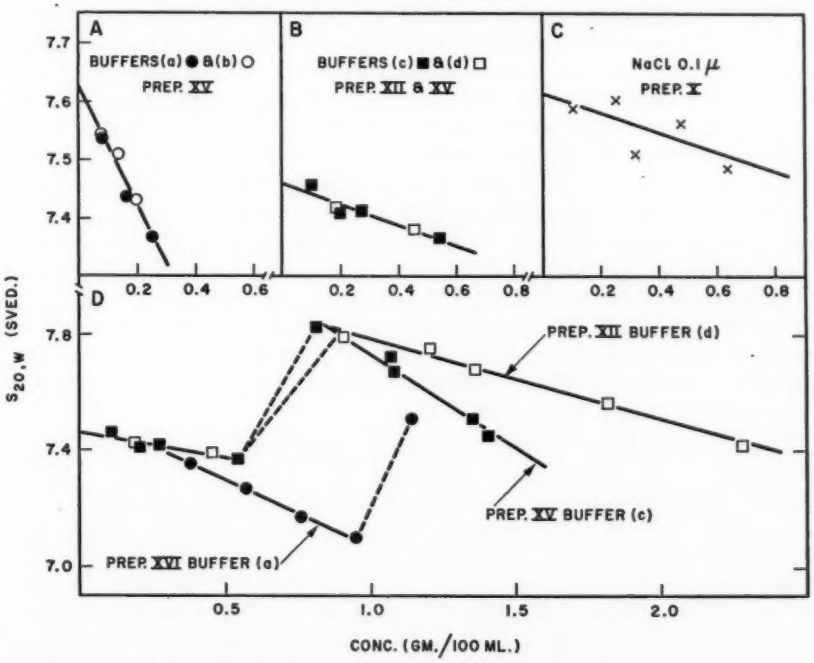

FIG. 2. Sedimentation behavior of γ-livetin.
A. In buffers (*a*): glycine, 0.1 μ, pH 9.5; and (*b*): veronal, 0.1 μ, pH 8.5.
B. In buffers (*c*): glycine, 0.2 μ, pH 9.5; and (*d*): veronal, 0.2 μ, pH 8.5.
C. In sodium chloride, 0.1 μ, pH *ca.* 6.5.
D. Showing anomalies at higher protein concentrations.

one series of measurements, as shown by the broken lines, and occurred with three different preparations in three different buffers. In view of this behavior all subsequent measurements were limited to the low concentration range where valid extrapolations could be made.

The results in the low concentration range, from which the $S^{\circ}_{20,w}$ values of Table I were obtained, appear in Fig. 2A, 2B, and 2C. In this range the concentration dependence is normal and small, giving a linear relation between s and c. The apparent differences in the slopes and intercepts are exaggerated by the open scale. Preparation XVI in 0.1μ glycine buffer (Fig. 2D) had an intercept about 0.1 S lower than Preparation XV in the same buffer (Fig. 2A) and this represents the maximum difference observed between preparations under comparable conditions. No difference between preparations is evident in Fig. 2B. The higher ionic strength buffers tended to give lower slopes and intercepts, but in general the differences between preparations and buffers were small.

Two diffusion measurements made at low concentrations in the two buffers gave similar results within experimental error. Molecular weights calculated from these measurements and appropriate sedimentation coefficients, as indicated in Table I, were not significantly different. One experiment performed at 0.85% protein concentration yielded a higher coefficient, as would be expected, but this result is considered doubtful in view of the anomaly in sedimentation behavior at this concentration. Had this value been used for extrapolation, the value of $D^{\circ}_{20,w}$ would be 4.35×10^{-7} cm.^{2}/sec., which is not appreciably different from the values used.

The measured partial specific volume and sedimentation and diffusion coefficients yielded a molecular weight of 1.5×10^5, the lowest observed by any of the methods employed. This is considered the most reliable estimate, since the heavy component, which separated during sedimentation, should have no appreciable effect on the sedimentation rate of γ-livetin. Similarly, the effect of this impurity on the partial specific volume and diffusion coefficient would be small, with any residual bias acting in the direction of exaggerating the molecular weight. The experimental errors in measuring these coefficients were small in the low concentration range.

The light-scattering method yielded the highest molecular weight, $(2.2\pm 0.07)\times10^5$, and a dissymmetry coefficient less than 1.05, indicating an essentially spherical molecule. In spite of the small standard error, this estimate is uncertain. γ-Livetin is so labile that some aggregates may have been formed during the final filtration, and these, together with the heavier impurity, would increase the average molecular weight both directly and indirectly since the method has a biased sensitivity to the larger particles. Osmotic pressure measurements, undertaken in an attempt to reconcile these divergent estimates of size, yielded a molecular weight of 2×10^5. These were likewise uncertain, since the osmotic heads were small and did not reach a true equilibrium, showing instead a slight but continuing decrease in head, which suggests aggregation.

Using the molecular weight obtained from the sedimentation and diffusion measurements and the intrinsic viscosity, the Scheraga–Mandelkern equation (10) gave a β-coefficient of 2.37×10^6, indicative of an axial ratio between 8 and 9. Conversely, if a spherical molecule, which is not precluded by the light-scattering measurements, is assumed, the value of the β-coefficient applicable to a sphere yields a maximum molecular weight of 1.86×10^5.

Discussion

This study indicates that γ-livetin is a labile protein that interacts and aggregates readily, and exhibits a detectable heterogeneity that is too small to permit ready separation of the fractions, interaction products, or other proteins involved. In these general properties, and in some specific chemical and physical attributes, γ-livetin resembles the γ-globulins of serum that, in spite of extensive investigation (2, 4), are still less adequately characterized than many other proteins.

All the preparations contained a small amount (5% or less) of a faster sedimenting component. This is probably lipovitellin since the other livetins or phosvitin should have been detectable electrophoretically. The alternative possibility that the faster sedimenting component is an aggregate would seem to be precluded by its presence in similar amount in a degraded sample dissolved in formic acid.

The difficulties experienced in preparing γ-livetin have made it impossible to estimate the amount present in egg yolk although the relative areas in electrophoretic and sedimentation diagrams of whole egg yolk (8, Fig. 2) suggest that it may constitute nearly half of the livetin fraction. It seems highly probable that previous preparations of lipovitellin, particularly those prepared from sodium chloride solutions, have contained γ-livetin. This hypothesis receives support from the observations of others (3, 16), which indicate that lipovitellin is heterogeneous. From electrophoretic studies Sugano (13) claims that two components are present in lipovitellin. It seems probable that the component he has termed α-lipovitellin is γ-livetin but this cannot be said with certainty on the basis of his electrophoretic studies on preparations made by methods that could alter γ-livetin or render it insoluble. In Sugano's (13) electrophoretic patterns of ether-extracted whole egg yolk the peak marked α-lipovitellin is in a similar position to the peak attributed to γ-livetin (8), although the reported mobility is higher. The lipid content of α-lipovitellin is low and corresponds more closely to γ-livetin than lipovitellin.

While the physical measurements showed that γ-livetin was not markedly concentration-dependent in the usual sense, the sedimentation rate showed an anomalous rise over a limited concentration range with normal concentration behavior at higher or lower concentration. This suggests an interaction that either increases the size of the kinetic units or reduces the frictional effects, or a combination of both. This anomalous behavior was not apparent in the other measurements, but most of these were confined to the low concentration range.

The estimates of the molecular weight varied from a minimum of 1.5×10^5 by sedimentation–diffusion methods to 2.2×10^5 obtained by light-scattering procedures, with other less satisfactory measurements yielding intermediate values. Similar values have been reported by the same methods for the γ-globulins of serum (2). While the random experimental errors were small for both the above methods, the light-scattering procedure is felt to be biased toward high values for reasons given earlier. A maximum molecular weight of 1.86×10^5 is indicated by the Scheraga–Mandelkern equation (10) from the sedimentation and intrinsic viscosity coefficients, if a spherical shape is assumed. The molecular weight of 2.2×10^5 obtained from light-scattering measurements would require a β-coefficient less than the theoretical for a sphere. The hydrodynamic methods are therefore consistent with one another for molecular weights between 1.5 and 1.8×10^5 with an axial ratio between 8 and unity respectively.

The results obtained clearly distinguish γ-livetin from the other proteins so far described in egg yolk. Apart from differences in solubility which form the basis of preparation, differences are evident in its electrophoretic, or sedimentation behavior. The low lipid and phosphorus contents of γ-livetin (and they may arise from impurities) differentiate it from the lipoproteins and phosvitin. It has a lower molecular weight than lipovitellin (14) and is distinguished from the other livetins by having at least twice the molecular weight of α-livetin and four times that of β-livetin.

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THE PHYSIOLOGICAL ACTIVITY OF LAMINARIN SULPHATE[1]

W. W. Hawkins and Verna G. Leonard

Abstract

When laminarin sulphate was injected intravenously into dogs it acted as an antithrombic agent. As such it was about one-third as potent as heparin. It also showed anticoagulant activity when administered subcutaneously and intramuscularly to the dog.

After the intravenous administration of a moderate amount of laminarin sulphate or of heparin to the dog, considerable anticoagulant material passed into the urine. When large oral doses were given to the rat, absorption from the intestine was very small, and much of the material passed into the faeces.

When it was given intravenously, laminarin sulphate acted like heparin in clearing alimentary lipaemia in the dog and the rat.

Introduction

The preparation of laminarin sulphate (1) and its effect on the clotting time of whole blood (2, 3) have been previously described. A more detailed investigation of the anticoagulant activity of laminarin sulphate and of some of its other physiological properties are reported in this paper. These include some studies on its absorption and excretion, and some preliminary experiments on its activity as an antilipaemic agent. For purposes of comparison similar studies were made on heparin.

Materials

Heparin sodium (Connaught), with a potency of 100 I. U./mg., was used. Three different lots of the sodium salt of laminarin sulphate were used during the course of the experiments. Each contained about 1.7 sulphate groups per glucose unit. *In vivo* tests with dogs showed that the preparations all had the same anticoagulant potency.

Immediately before injection the anticoagulants were dissolved in physiological saline, which was prepared from water freshly distilled from glass.

Solutions of Salts

All aqueous solutions were prepared in water from an all-glass still. Sodium chloride (Merck reagent for biological use) in a concentration of 0.85% was used for work both *in vivo* and *in vitro*. The other solutions were calcium chloride 0.02 *M*, and sodium citrate 3.8%.

Thromboplastin

Difco thromboplastin powder was extracted with saline at 45–48° C. for 10 minutes in proportions of 0.3 g. to 8 ml. of saline, and the mixture centrifuged. The undiluted supernatant fluid was used. It was frozen in lots of 1 or 2 ml., and thawed just before use. It did not lose activity in the frozen state.

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Thrombin

A stock solution containing 200 U. (National Institutes of Health) per ml. was prepared from Parke-Davis topical thrombin in a 1:1 mixture of glycerol and saline. It was kept in the refrigerator, and maintained its activity for a long period. For use it was diluted 1 in 10 with saline.

Anticoagulant Antagonists for Injection

Protamine sulphate, 1% Lilly, was diluted with saline to a concentration of 50 mg. in 25 ml.

A 5% emulsion of vitamin K_1 (Mephyton, Merck) was diluted with warm saline to a concentration of 50 mg. in 30–50 ml.

Methods: Blood Clotting Efficiency

Collection of Blood and Preparation of Plasma

Venous blood was drawn into an oiled syringe. After samples had been taken for measurement of the clotting time, most of the remaining portion was mixed with one-ninth its volume of sodium citrate solution. This was centrifuged at 1500 r.p.m. for 15 minutes, and the plasma pipetted off. It was refrigerated until the tests were done on it a few hours later.

Condition of Tests

All of the preparations used in the tests of blood clotting efficiency were brought to 37° C., and all the tests were carried out at that temperature.

Pyrex tubes 1 cm. × 7.5 cm. were used.

The clotting time of whole blood was measured to the nearest second. For convenience it has been expressed to the nearest 1/10th of a minute. If it exceeded half an hour it was usually not followed further, because in such cases the degree of coagulation which was taken as the end point rarely occurred.

In the tests on plasma the appearance of fibrin was taken as the end point. Measurements of prothrombin and thrombin times were usually not carried beyond 30 seconds, and of recalcification time not beyond 3 minutes, because the small amount of fibrin in such cases did not produce a clear end point.

Clotting Time

The clotting time of whole blood was measured by the method of Lee and White (4), using three dry tubes with approximately 1 ml. of blood in each.

Recalcification Time

To 0.1 ml. of plasma 0.2 ml. of calcium chloride solution was added. The mixture was examined in 30 seconds, then at intervals of 15 seconds.

Prothrombin Time

The technique was the one-stage method of Quick (5). To 0.1 ml. of plasma 0.1 ml. of thromboplastin solution and 0.1 ml. of calcium chloride solution were added. The calcium chloride was added last by blowing in from a pipette, and timing was started from its addition.

Thrombin Time

To 0.2 ml. of plasma 0.1 ml. of saline and 0.1 ml. of thrombin solution were added in that order. The time was taken from addition of the thrombin.

TABLE I

THE CLOTTING EFFICIENCY OF THE BLOOD OF DOGS AFTER INTRAVENOUS INJECTION OF HEPARIN OR OF LAMINARIN SULPHATE

Time after injection (hr.)	Clotting time (min.)		Re-Ca^{++} time (sec.)		Prothrom. time (sec.)		Throm. time (sec.)	
	Hep.	Lam-SO_4	Hep.	Lam-SO_4	Hep.	Lam-SO_4	Hep.	Lam-SO_4
(Initial)	6.5–10.3 8.6		30–75 60		5–10 7		7½–11½ 8¾	
½	26.1–>30	21.4–>30	90–>180	120–>180	5½–12 9¾	5½–13½ 9	15½–35 28	17–30 24½
1	14.7–27 18.5	16–26 20	75–135 105	105–>180	6–10½ 8	5½–12 8	9½–20½ 14	15½–26 21
2	9–13.3 11.9	9–17.3 13.8	45–135 81½	60–>180	5–10 7½	5¼–11½ 8¼	8–16 9¾	13–20½ 16½
3	8.2–12.2 10	8–13.3 10.2	60–75 65	60–105 90	6½–9½ 8¼	5¾–10½ 8¼	7½–8 8	12–20½ 16
4	7.6–8.6 8.2	8.1–11.2 10.2	60–75 65	75–75 75	7½–9 8½	6–10 8	8–9½ 9	11–19½ 14½

Doses: heparin 1 mg./kg., laminarin sulphate 3 mg./kg.
Experiments on seven dogs, each of which received one injection of each anticoagulant.
Values are expressed as ranges and averages.

Experimental Procedures and Results

Anticoagulant Effect on the Blood of the Dog in Vivo

Heparin and laminarin sulphate were given in doses of 0.5 to 6 mg./kg. of body weight to 16 dogs. The dose per kg. was contained in 0.5 ml. of saline. In most cases the doses were 1 mg. of heparin and 3 mg. of laminarin sulphate per kg. Table I shows the results of experiments on seven dogs, each of which was given an intravenous injection of each anticoagulant.

The immediate effect on the blood clotting efficiency, as measured ½ hour after injection, indicated that laminarin sulphate is one-third as potent as heparin.

The thrombin time was affected more than any other measure of the clotting efficiency of the plasma. This indicates that laminarin sulphate, like heparin (6, 7), acts in an important capacity in the terminal stage of clotting as an antithrombin. To confirm this point some experiments were done in which the normal prothrombin and thrombin times were brought nearer the same value by using for their measurement a more dilute solution of thromboplastin and a more concentrated solution of thrombin respectively. After these modifications heparin and laminarin sulphate still caused greater changes in the thrombin time than in the prothrombin time, and laminarin sulphate in a dose initially equivalent to heparin still had a more prolonged antithrombic effect.

The effect of intramuscular and subcutaneous administration of the anticoagulants was studied, using twice the usual intravenous doses. In these experiments only the blood clotting times were followed. Table II shows the results on three dogs. When given by these routes both heparin and laminarin sulphate showed considerable variation in both the quantitative effect on the clotting time and the duration of activity. The maximum clotting time appeared later than it did after intravenous injection, and the effect was more prolonged. After intramuscular injection the maximum clotting times were of the same order as those after the intravenous injection of half the dose. Absorption was less efficient after subcutaneous than after intramuscular injection. The data in Table II indicate that when it is given by one of these routes, a dose of laminarin sulphate initially as potent as heparin generally shows a more prolonged activity.

Comparison with Dicumarol, and the Effect of Antagonists

The effects of heparin and of laminarin sulphate on the various measures of blood clotting efficiency were compared with those of dicumarol. The efficacy of vitamin K_1 and of protamine as antagonists was also investigated. The results of one such experiment are shown in Table III.

There are two noteworthy points. One is that in the case of dicumarol the major effect is on the prothrombin time, which is consistent with the concept of its activity as a vitamin K antagonist (8), and consequently an antiprothrombin. Laminarin sulphate, like heparin, affects primarily the thrombin time, and is presumably therefore principally an antithrombin. The other point to be noted is the short time in which laminarin sulphate

TABLE II

THE EFFECT OF INTRAMUSCULAR AND SUBCUTANEOUS INJECTIONS OF HEPARIN AND OF LAMINARIN SULPHATE ON THE CLOTTING TIME OF THE BLOOD OF DOGS

Time after injection (hr.)	Intramuscular		Subcutaneous	
	Heparin	Lam-SO_4	Heparin	Lam-SO_4
	Clotting time in minutes			
Initial	6.3–10.9 8.2			
½	14–16.9 15.5	14.1–16.9 15.5		7.6–18.5 11.9
1	15.8–18.7 17.2	17.4–24.4 20.9	8.1–14.1 11.5	10.3–21.2 16.1
2	20.2–28.4 24	24.8–28.7 26.4	14.9–16.2 15.6	14.3–22.3 19
3	16.2–>30	28.7–30.1 29.3	11.6–19.5 16.2	18.8–25 21.2
4	13.3–21.2 17.6	20.6–24.1 22.4	10.7–14.7 10.8	15.1–18.4 17.1
6	7.7–11.6 9.3	8.5–17.3 12.5	7.6–10.9 9.5	11.5–15.4 13.5

Doses: heparin 2 mg./kg., laminarin sulphate 6 mg./kg.
Experiments on three dogs, each of which received one subcutaneous and one intramuscular injection of each anticoagulant.
Values are expressed as ranges and averages.

TABLE III

COMPARISON OF THE ANTICOAGULANT ACTIVITY OF DICUMAROL WITH THAT OF HEPARIN AND LAMINARIN SULPHATE, AND THE EFFECT OF ANTAGONISTS

Time	Treatment	Clotting time (min.)	Re-Ca^{++} time (sec.)	Prothrom. time (sec.)	Throm. time (sec.)
Day 1:	Dicumarol, 30 mg./day for 4 days, by mouth	7.5	60	5½	7
Day 4: 10.00 a.m.		10	75	20	8
11.30	Vit. K_1, 100 mg., intravenously				
1.30 p.m.		10.5	60	14	7
4.30		9	60	6	7
Day 10: 9.30 a.m.		6	60	7	9½
9.45	Heparin, 1 mg./kg., intravenously				
10.00		34	210	7½	>30
10.01	Protamine sulphate, 1.2 mg./kg., intravenously				
10.21		7.5	75	7½	11½
10.41		6.5	75	7	9½
Day 13: 10.00 a.m.		8	45	5	6
10.15	Laminarin sulphate, 3 mg./kg., intravenously				
10.30		24	150	6	10
10.40	Protamine sulphate, 2.5 mg./kg., intravenously				
10.58		8	75	5	7½

NOTE: All experiments on the same dog.

or heparin can be neutralized by protamine, in comparison to the time required for the adequate control of dicumarol by vitamin K_1. The administration by mouth of large doses of Menadione before trials on dogs, and the intravenous injection of vitamin K_1 during trials did not alter the effect of laminarin sulphate.

These results offer further evidence of the similarity of laminarin sulphate to heparin in its mode of action as an anticoagulant.

Absorption and Excretion

Toluidine blue shows a spectral absorption band at λ 620 mμ. In the presence of heparin the color changes to purple, and a new band appears at a shorter wavelength. This reaction has been made the basis of a method for the estimation of heparin in biological materials (9, 10, 11). We found that laminarin sulphate has the same metachromatic activity, and more recently Dewar (12) has published results of the use of the reaction in the quantitative estimation of laminarin sulphate. We have used the reaction as a qualitative test and for rough quantitative estimations of heparin and laminarin sulphate in blood plasma, urine, intestinal contents, and faeces.

Urinary Excretion by the Dog after Intravenous Injection

An experiment was done to determine whether after the intravenous injection of large doses of heparin and laminarin sulphate any anticoagulant material is excreted in the urine.

At the beginning of the experiment the blood clotting times of two dogs were 7 minutes and 7.5 minutes. One was given heparin and the other laminarin sulphate, 20 mg./kg. Urine was collected from the first dog for 12½ hours and from the second for 6 hours. The alcohol-insoluble material from the urine was dissolved in saline. In each case a sample of the solution gave a purple color with toluidine blue. The rest of the solution was injected intravenously. One-half hour after injection the clotting times of the blood were respectively 24 and 14.5 minutes. Previous to the experiment urine was collected from each dog, and its alcohol-insoluble fraction tested in the same way. It did not in either case show metachromatic activity with toluidine blue, or affect the clotting time of the blood when injected intravenously.

Absorption and Excretion by the Rat after Oral Administration

Rats were fasted for 18 hours. Aqueous solutions of heparin and of laminarin sulphate were given by stomach tube in doses of 100 and 200 mg. per 100 g. of body weight. After 1–4 hours the animals were anaesthetized, the thorax opened, and blood taken from the exposed heart. The non-protein alcohol-insoluble fraction of the plasma was tested for metachromatic activity with toluidine blue. Measurements of clotting efficiency were considered invalid because of the severe trauma imposed. Urine and faeces were collected from some of the animals for 24 hours following administration of the anticoagulant. The animals were then killed. The small intestine was removed, and its contents collected. The large intestine with the rectum was removed, and the contents combined with the faeces. The alcohol-insoluble substance of each fraction was tested for metachromatic activity with toluidine blue.

In some of the animals the anticoagulants were detected in the plasma, particularly after the larger dose of laminarin sulphate. In a few cases they were detected in the urine. In most cases they were detected in the contents of the intestines and the faeces. Both anticoagulants were apparently poorly absorbed from the intestine. When material from plasma showed metachromatic activity with toluidine blue after the larger dose of laminarin sulphate, the color intensity was approximately the same as that given by material from the same amount of plasma to which 0.5 mg./100 ml. had been added. This would mean that about 0.02% of the administered dose was in the circulation. The concentration of laminarin sulphate in the blood of dogs must be 3–4 mg./100 ml. to triple the clotting time. It would therefore be impracticable, if not impossible, effectively to use laminarin sulphate by the oral route in anticoagulant therapy. In this respect again it resembles heparin.

Effect on Alimentary Lipaemia

Fasting rats were given by stomach tube 1 ml. of corn oil per 100 g. of body weight. Three hours later they were given intravenously 0.1 mg. of heparin or 0.3 mg. of laminarin sulphate in 0.5 ml. of saline per 100 g. One-half hour after the injection blood was taken from the heart, and the lipid content of the plasma estimated by the method of de la Huerga *et al.* (13). The results are shown in Table IV.

TABLE IV

THE EFFECT OF HEPARIN AND OF LAMINARIN SULPHATE ON ALIMENTARY LIPAEMIA IN RATS

Material injected	No. of rats	Plasma lipid, mg./100 ml.	
		Range	Average
Saline	5	157 – 324	220
Heparin	5	138 – 150	144
Laminarin sulphate	6	106 – 217	175

A similar experiment was done on two dogs. After a sample of blood had been taken from the fasting animal, the animal was given a meal of 8 g. of whole-milk powder and 5 ml. of corn oil per kg. of body weight, mixed in warm water. Three hours later a sample of blood was taken, and an intravenous injection was given of 1 mg. of heparin or 3 mg. of laminarin sulphate in 0.5 ml. of saline per kg. of body weight. Blood was taken at intervals up to 2 hours after the injection. The degree of lipaemia of the plasma was estimated from the optical density at λ 650 mμ. The results are shown in Table V.

The results of both experiments showed that laminarin sulphate has an effect similar to that of heparin in clearing alimentary lipaemia.

TABLE V

THE EFFECT OF HEPARIN AND OF LAMINARIN SULPHATE ON ALIMENTARY LIPAEMIA IN DOGS

Material injected	No. of expts.	Optical density of plasma at λ 650 mμ										
		Before meal	3 hr. after meal		Time after injection (hr.)							
					¼		½		1		2	
		Range	Range	Av.	Range	Av.	Range	Av.	Range	Av.	Range	Av.
Saline	1	.060–.060		.760		.940		1.500		.485		.420
Heparin	1			.790		.105		.138		.052		
Laminarin sulphate	4		.227–.902	.495	.156–.313	.235	.212–.268	.238	.131–.197	.149	.062–.182	.102
Saline	3	.029–.030	.333–.692	.562	.280–.780	.496	.411–.615	.529	.545–.701	.601	.374–1.090	.805
Heparin	1			.634		.040		.048		.049		.900
Laminarin sulphate	2		.840–1.080	.960	.188–.249	.219			.178–.194	.186	.140–.207	.174

Discussion

The similarity of laminarin sulphate to heparin in its physiological properties is shown in its effect on blood coagulation, its behavior in the intestinal tract, and its antilipaemic action.

Anticoagulant Activity and Potency

From the chemical nature of laminarin sulphate it would be expected to affect the same stage or stages in the blood clotting process as does heparin. Similar sulphated polysaccharides have been found to act in the same way (14, 15, 16). There is, however, a great variation in their potency as anticoagulants. Thus dextran sulphate has been shown by some authors to have one-fifth the activity of heparin (15), and by others one-seventh (17). Sulphated pectin showed 1/10 (16), and treburon* 1/10 to ¼ (18, 19) the activity of heparin. One group of investigators (19) found that this whole range of values for treburon was obtainable by varying the assay method. When one of our preparations of laminarin sulphate was tested by the *in vitro* method for heparin in current use at the Connaught Medical Research Laboratories, it was found to have about one-fifth the activity of heparin, but when we injected it into dogs it showed about one-third.

We have had one preparation of laminarin sulphate which was nearly half as potent as heparin, but most of them have shown about one-third when tested in the same way *in vivo*. Other authors (3, 12) have reported this range of potency for preparations of the compound with the same sulphate content. Laminarin sulphate appears to be one of the most potent anticoagulants of the polysaccharide type.

Absorption and Excretion

Mangieri *et al.* (19) could obtain no evidence that treburon is absorbed from the intestine of the rabbit after very large oral doses.

We have found that laminarin sulphate, like heparin, is absorbed to a very small extent from the intestine of the rat, and also, like heparin, is excreted in the faeces after a large oral dose. Fischer and Astrup (20) demonstrated this in mice with heparin. It may be that some of the material is degraded during passage through the intestine, as Åberg (21) found in the case of dextran.

After the intravenous injection of relatively large doses it has been shown that treburon is excreted in the urine by rabbits (19), and dextran sulphate (17) and heparin (22, 23) by men (17, 22) and dogs (23). At least the urine in these cases was found to contain anticoagulant material. It has been shown, however, that heparin loses potency in passing through the body (24), which could be due to the action of heparinase (25, 26). This enzyme also acts upon treburon (26), and it is possible that it or a similar enzyme will degrade other sulphated polysaccharides. Our experiments demonstrated that laminarin sulphate, or at least an active derivative of it, can also pass from the blood into the urine.

* Sulphated polygalacturonic acid methyl ester methyl glycoside.

Antilipaemic Activity

We have shown that laminarin sulphate reduces the turbidity of alimentary lipaemia in rats and dogs. Besterman and Evans (27) have demonstrated that it acts similarly in humans toward hyperlipaemia in ischaemic heart disease. In this respect it resembles heparin. There is very little information of a quantitative nature on this aspect of its activity.

Further work on the antilipaemic properties of laminarin sulphate is in progress in this laboratory.

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THE METABOLISM OF ANIMAL TISSUES CULTIVATED IN VITRO

II. AMINO ACID METABOLISM OF CHICK EMBRYONIC KIDNEY, CHICK EMBRYONIC LIVER, AND MONKEY KIDNEY CORTEX CULTURES[1]

ARTHUR E. PASIEKA, HELEN J. MORTON, AND JOSEPH F. MORGAN

Abstract

Freshly-explanted chick embryonic kidney, chick embryonic liver, and trypsinized monkey kidney cortex cells have been cultivated *in vitro* in completely synthetic medium M 150. The amino acid changes in the nutrient medium during cultivation of these tissues have been studied by paper chromatography. A characteristic pattern of amino acid uptake and accumulation in the used culture medium has been demonstrated with each type of tissue culture. It has also been shown that, while the amino acid changes in the medium are different with each type of tissue culture, all cultures examined removed adenine from the medium and liberated small amounts of material thought to be hypoxanthine.

Introduction

A previous study from this Laboratory (22) has shown that freshly-explanted chick embryonic heart tissues cultivated *in vitro* in a completely synthetic medium (13,17) caused marked changes in the amino acid content of the culture fluid. Cystine, and the basic amino acids lysine, histidine, and arginine were almost completely removed from the medium. Less complete removal was observed with valine, phenylalanine, tryptophan, isoleucine, and leucine. Concurrently, increases in the concentration of glutamine, serine, glycine, threonine, alanine, and methionine were detected.

The investigation with chick embryonic heart cultures (22) established a definite pattern of amino acid changes in the culture medium but did not indicate whether this pattern could be related specifically to the tissue under cultivation. Since comparable metabolic studies on cultures of other tissues are lacking in the literature, it was considered of value to extend these investigations to a variety of other cell systems. In view of the demonstration (10) that C^{14}-labelled adenine is rapidly incorporated by tissue cultures, determinations of this purine base were included in the study. It is hoped that the metabolic patterns reported in the present communication may provide a basis for the biochemical characterization of different cells in tissue culture.

Materials and Methods

Tissue Culture Procedures

Cultures were prepared from the tissues of 11-day-old chick embryos. The kidneys and livers were dissected out separately under aseptic conditions and pooled lots of 10 to 15 organs used for each experiment. The tissue was chopped to a fine pulp with curved scissors and portions were transferred to the culture vessels by a capillary pipette. After a period of approximately

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30 minutes, during which the tissue became fixed directly to the glass without the use of plasma clots, the synthetic medium was added and the cultures were incubated at 37° C. Each culture contained from 10 to 20 mg. of tissue wet weight (9), the amount previously found to be optimal for amino acid metabolism studies with chick embryonic heart tissues (22). All cultures were prepared in T-30 flasks (4) and received 5.0 ml. of synthetic medium per flask.

Monolayer tissue cultures were prepared from the cortex of kidneys obtained from *Rhesus* monkeys. The tissue was chopped to a fine pulp with curved scissors and treated with trypsin to disperse the cells into a suspension (32). The trypsin was removed by washing in saline, the cells were centrifuged, and resuspended in synthetic medium M 150 to a concentration of approximately 1,000,000 cells per ml., as determined by direct counts in a hemocytometer. Each T-30 flask was then seeded with 2.0 ml. of this cell suspension. It was found experimentally that a 2.0 ml. volume was just sufficient to cover the bottom of the flasks with a thin film of nutrient and provided conditions that facilitated adhesion of the individual cells to the glass surface. After overnight incubation at 37° C., the kidney cells were firmly attached and an additional 3.0 ml. of medium M 150 was added. At all subsequent renewals of nutrient, 5.0 ml. volumes were used.

The synthetic medium used throughout these experiments was M 150 (13,17). This medium was prepared at frequent intervals and sterilized by passage through UF fritted glass filters. The culture medium was renewed twice a week and all cultures were examined microscopically at the time of the fluid renewals to detect signs of degeneration. Only cultures of normal morphological appearance were used for the amino acid metabolism studies. From these cultures, the samples of "used" culture medium for chromatographic analysis were obtained at the time of regular fluid renewal.

Chromatographic Techniques

Changes in the amino acid composition of medium M 150 brought about by the tissue cultures were measured by paper chromatography. The used nutrient fluids from groups of cultures were pooled and 5.0 ml. samples used for analysis. These were concentrated to dryness *in vacuo* over sulphuric acid and reconstituted in 0.2 ml. of deionized water. Five to twenty microliter quantities of this concentrated material were found sufficient for satisfactory analyses, with one-dimensional descending paper chromatograms.

Two solvent systems were employed in these studies: a *n*-butanol–acetic acid–water mixture, and an ethanol–butanol–water mixture. The exact composition of these two solvent systems has been described previously (22). Schleicher and Schuell No. 597 paper was found most suitable for the acidic solvent system, and Whatman No. 1 paper for the neutral solvent. Under the conditions developed for the use of these two solvents, desalting of the concentrated media was not found necessary.

All chromatograms were routinely developed at room temperature for 16 to 18 hours and then dried at 110° C. for 2 to 3 minutes. It was observed that separation and resolution could be increased by developing for a second

18 hour period in the same solvent system before applying the chromogenic reagents. In some cases, especially when the neutral alcoholic solvent system was used, a third and fourth development period was found useful. Drying the chromatograms between the development periods was found to yield a more effective separation of the amino acids than could be obtained by one continuous 36 to 72 hour development.

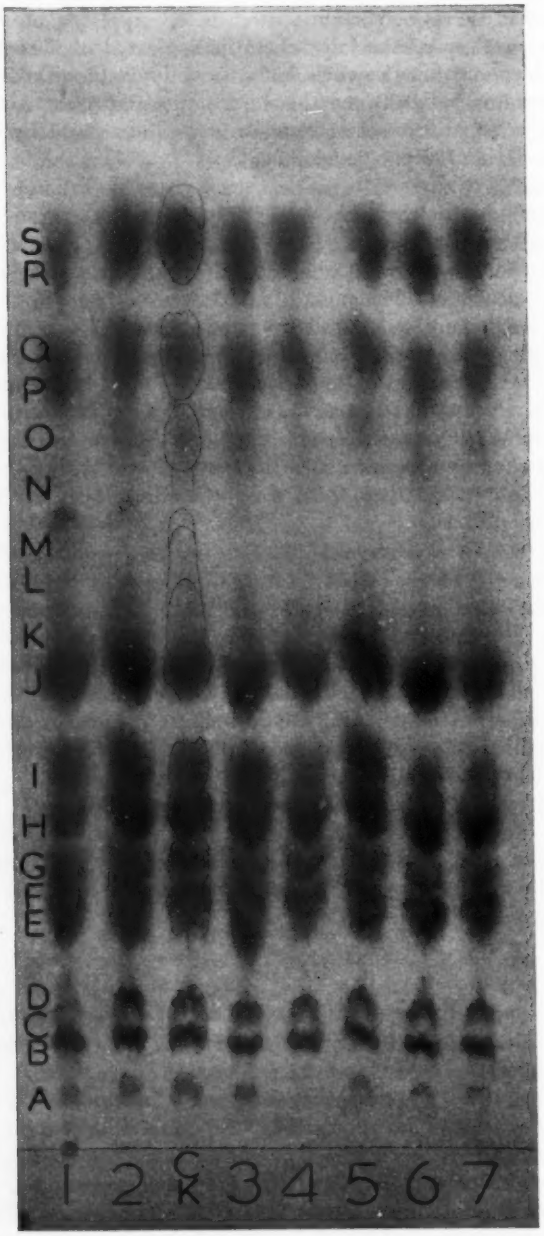
Ninhydrin-positive material was detected by spraying the chromatograms with 0.2 to 0.4% ninhydrin dissolved in either water-saturated *n*-butanol or 85% aqueous ethanol. The isatin reagent of Acher, Fromageot, and Jutisz (1) as modified by Saifer and Oreskes (26) was also used as a general chromogenic reagent, for amino acids. The most useful procedure was to dip the chromatograms in the isatin solution, allow excess reagent to run off, and heat in an oven at 100–110° C. for 10 to 15 minutes. When used under these conditions, the isatin reagent was found to be particularly valuable for detecting proline, glutamic acid, glutamine, aspartic acid, and lysine. Each of the amino acids present in M 150 was dissolved in the basic salt solution and run individually on paper chromatograms, with the two solvent systems employed, in order to establish its relative position on the chromatograms under these experimental conditions.

In addition to the two general methods used for amino acid detection, specific color reactions were employed for a number of amino acids. Cystine and methionine were measured by the platinic iodide method of Toennies and Kolb (28). Arginine was determined by the 8-hydroxyquinoline reagent of Jepson and Smith (7) or by the Sakaguchi reaction as modified by Williams (30). All chromatograms were checked for the presence of ornithine by the isatin and *p*-dimethylaminobenzaldehyde reagent of Jepson and Smith (7) and by the vanillin reagent of Curzon and Giltrow (3). Histidine and tyrosine were determined by the Pauly reaction, as modified by Block (2). Tryptophan was estimated by Ehrlich's reagent, as modified by Smith (27), and by the perchloric acid method of Giri (5). Specific color reactions developed in this Laboratory were used for the determination of proline (20), phenylalanine (18,21), and hydroxyproline (19). Purine bases and inorganic salts were measured by the chloride method of Wood (31).

Results

Amino Acid Changes during Cultivation of Chick Embryonic Kidney Tissue

During the cultivation of freshly explanted chick embryonic kidney tissues in M 150, samples of the used culture medium were removed, concentrated, and analyzed on paper chromatograms for changes in amino acid composition. Samples from each lot of M 150 used on the cultures were concentrated to dryness at the time of preparation and included on the chromatograms as controls. A typical chromatogram, showing the degree of separation and resolution of the amino acids in medium M 150, is presented in Fig. 1. This chromatogram has been developed in the *n*-butanol – acetic acid – water solvent system and subsequently treated with ninhydrin (22).


S
R
Q
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O
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M
L
K
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H
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1
2
C
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3
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7

TABLE I

CHANGES IN AMINO ACID COMPOSITION OF SYNTHETIC MEDIUM M 150 DURING CULTIVATION OF CHICK EMBRYONIC KIDNEY TISSUE[a]

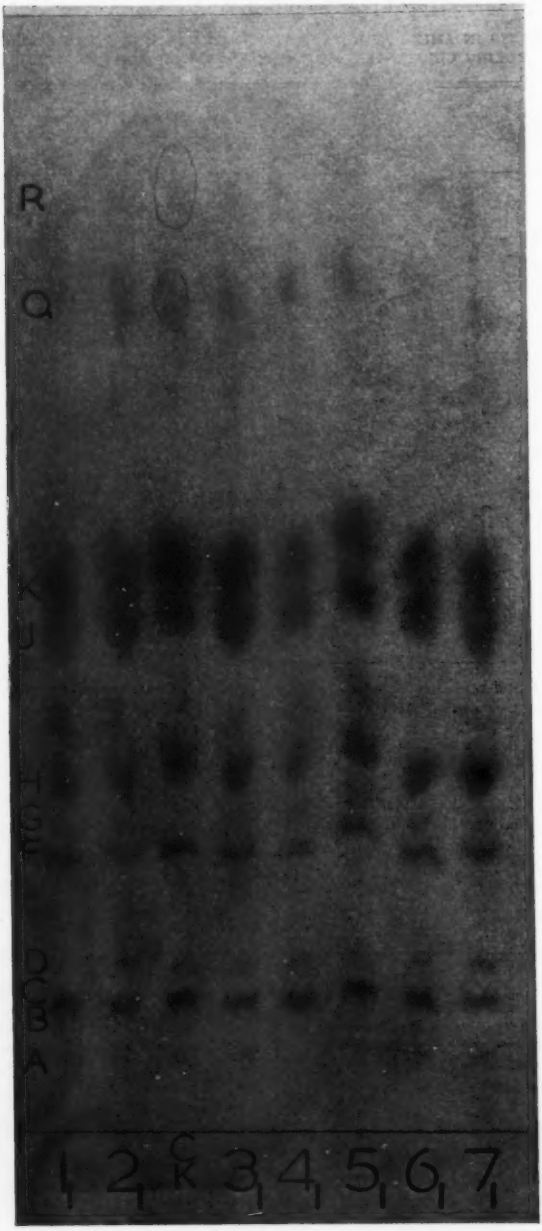
Amino acid	Age of culture in days[b,c]				
	5	8	12	20	28
Cystine	**	*	*	**	**
Lysine	+	++	0	0	*
Histidine	**	0	*	0	0
Arginine	*	*	*	*	*
Glutamine	0	++	++	++	+
Aspartic acid	0	0	0	0	0
Serine	+	+	+	+	+
Hydroxyproline	0	0	0	0	0
Glycine	+	+	+	+	+
Glutamic acid	*	+	++	0	0
Threonine	+	++	++	+	+
Alanine	++	++	++	+	+
Proline	*	0	0	*	*
Tyrosine	*	0	0	*	*
Tryptophan	**	*	*	*	*
Methionine	*	++	++	+	0
Valine	*	+	+	*	*
Phenylalanine	*	0	*	*	*
Isoleucine	**	0	0	*	*
Leucine	**	0	0	*	*

[a] All changes listed represent alterations from unused M 150 controls.
[b] All media remained in contact with the cultures for 3- or 4-day periods.
[c] + = slight increase, ++ = moderate increase, * = slight decrease, ** = moderate decrease, 0 = no change.

The use of isatin as a general amino acid reagent is illustrated in Fig. 2. This chromatogram, as in the case of Fig. 1, has been developed in *n*-butanol – acetic acid – water. Use of the isatin procedure was found to complement that of ninhydrin in detecting the pattern of amino acid changes caused by the living tissues.

FIG. 1. Changes in amino acid composition of synthetic medium M 150 during cultivation of chick embryonic kidney tissues. Chromatogram run in *n*-butanol – acetic acid – water solvent system and developed with ninhydrin. CK = unused M 150 control. Numbers 1 to 7 = successive changes of medium exposed to the cultures for 3 days before each change.

S = Isoleucine
R = Leucine
Q = Phenylalanine
P = Valine
O = Methionine
N = Tryptophan
M = Phenol red
L = Tyrosine
K = Proline
J = Alanine
I = Threonine
H = Glutamic acid
G = Glycine, serine, hydroxyproline
F = Aspartic acid
E = Glutamine
D = Arginine
C = Histidine
B = Lysine
A = Cystine


R
Q
K
J
H
G
F
D
C
B
A
1
2
K
3
4
5
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7

The changes revealed by application of the general amino acid reagents and by the use of neutral as well as acidic solvent systems were not marked and recourse to specific color reactions for various amino acids was found necessary. From these experiments, the changes in amino acid content of the medium during cultivation of chick embryonic kidney tissues were obtained and are summarized in Table I. A moderate uptake of cystine, tryptophan, and phenylalanine and a slight uptake of arginine, proline, tyrosine, isoleucine, and leucine was observed. Increases in the concentrations of glutamine, serine, glycine, threonine, and alanine were noted. No changes in the concentrations of aspartic acid or hydroxyproline could be detected. The values obtained for lysine, histidine, glutamic acid, methionine, and valine varied with different ages of cultures and consistent trends were not observed.

Amino Acid Changes during Cultivation of Chick Embryonic Liver Tissue

Culture fluids were removed from chick embryonic liver cultures, concentrated, developed on paper chromatograms, and analyzed by general and specific amino acid reagents, and the pattern of changes caused by tissue metabolism determined. The results of these experiments are presented in Table II. Slight to moderate uptake of cystine, arginine, tryptophan, phenylalanine, isoleucine, and leucine are evident. Slight to moderate increases in the concentrations in the medium of lysine, glutamine, aspartic acid, serine, glycine, threonine, alanine, and methionine were observed. No change in the hydroxyproline content of the medium was found, while the results with histidine, glutamic acid, proline, tyrosine, and valine were somewhat variable.

Amino Acid Changes during Cultivation of Trypsinized Monkey Kidney Cells

The used fluids from monolayer cultures of trypsinized monkey kidney cortex cells were collected and analyzed, as described previously, and the results are summarized in Table III. Slight to moderate uptakes of cystine, proline, tyrosine, tryptophan, isoleucine, and leucine were observed. With lysine, histidine, arginine, and glutamine, moderate uptakes were found during the early stages of cultivation but these uptakes ceased after the 11th to 14th day. Increases in the medium of serine, glycine, glutamic acid, threonine, alanine, and methionine were observed throughout the experimental period. No changes in the concentrations of aspartic acid, hydroxyproline, or phenylalanine were detected at any time.

FIG. 2. Changes in amino acid composition of synthetic medium M 150 during cultivation of chick embryonic kidney tissue. Chromatogram run in *n*-butanol – acetic acid – water solvent system and developed with isatin. CK = unused M 150 control. Numbers 1 to 7 = successive changes of medium exposed to the cultures for 3 days before each change.

R = Leucine
Q = Phenylalanine
K = Proline
J = Alanine
H = Glutamic acid
G = Glycine, serine, hydroxyproline
F = Aspartic acid
D = Arginine
C = Histidine
B = Lysine
A = Cystine

TABLE II

CHANGES IN AMINO ACID COMPOSITION OF SYNTHETIC MEDIUM M 150 DURING CULTIVATION OF CHICK EMBRYONIC LIVER TISSUE [a]

Amino acid	Age of culture in days[b,c]				
	3	10	16	20	27
Cystine	*	**	**	**	**
Lysine	+	+	++	++	+
Histidine	**	0	*	+	*
Arginine	*	**	*	*	*
Glutamine	++	++	++	++	*
Aspartic acid	+	0	+	+	+
Serine	+	+	+	+	+
Hydroxyproline	0	0	0	0	0
Glycine	+	+	+	+	+
Glutamic acid	+	0	+	0	0
Threonine	+	++	++	++	0
Alanine	0	0	++	++	0
Proline	0	*	+	++	0
Tyrosine	*	*	0	0	0
Tryptophan	*	*	*	*	*
Methionine	0	0	++	++	+
Valine	0	*	0	+	*
Phenylalanine	**	**	*	*	*
Isoleucine	**	**	*	0	*
Leucine	**	**	*	0	*

[a,b,c] See Table I.

TABLE III

CHANGES IN AMINO ACID COMPOSITION OF SYNTHETIC MEDIUM M 150 DURING CULTIVATION OF TRYPSINIZED MONKEY KIDNEY CELLS[a]

Amino acid	Age of culture in days[b,c]				
	7	11	14	20	27
Cystine	**	**	*	*	**
Lysine	*	*	+	+	+
Histidine	**	*	*	0	0
Arginine	**	**	*	0	0
Glutamine	*	*	0	0	0
Aspartic acid	0	0	0	0	0
Serine	+	+	+	+	+
Hydroxyproline	0	0	0	0	0
Glycine	+	+	+	+	+
Glutamic acid	++	+	+	++	++
Threonine	+	++	+	++	++
Alanine	++	++	++	++	++
Proline	*	*	0	*	*
Tyrosine	*	*	*	*	*
Tryptophan	*	*	*	*	*
Methionine	+	+	++	+	++
Valine	*	*	+	+	*
Phenylalanine	0	0	0	0	0
Isoleucine	*	*	*	*	*
Leucine	*	*	*	*	*

[a,b,c] See Table I.

Changes in Purine Bases during Cultivation of Various Tissues

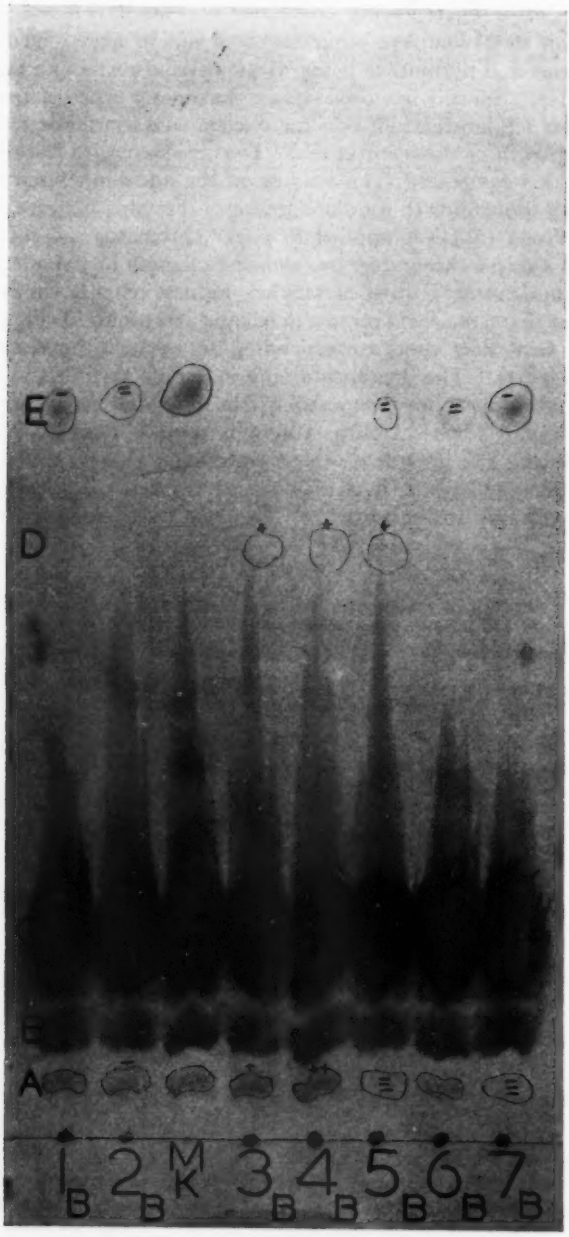
In addition to a complete supplement of amino acids, medium M 150 contains purine and pyrimidine bases, vitamins, and certain accessory growth factors (13,17). Since it has been shown that many types of tissue cultures readily utilize C^{14}-labelled adenine for nucleic acid synthesis (10) and that the rate of growth of these cultures can be correlated with the rate of incorporation of this compound (11), studies on the adenine content of medium M 150 during tissue growth were undertaken. For this purpose, the chloride method of Wood (31) was applied to paper chromatograms of the culture medium. A typical chromatogram, showing changes in the adenine content of the medium during growth of monkey kidney cells, is shown in Fig. 3. It is apparent that a marked decrease in adenine has occurred (Fig. 3, E region) and that a faint new spot, corresponding to hypoxanthine, has appeared (Fig. 3, D region). The densely-staining materials at the B and C regions of this chromatogram are composed of the inorganic salts present in the culture medium. Identical chromatograms were obtained with the fluids harvested during the growth of chick embryonic kidney, chick embryonic heart, and chick embryonic liver cultures, and a general uptake of adenine by all these cultures was indicated.

TABLE IV

COMPARATIVE PATTERN OF CHANGES IN AMINO ACID COMPOSITION OF SYNTHETIC MEDIUM M 150 DURING CULTIVATION OF VARIOUS TISSUES *in vitro*[a]

Amino acid	Chick heart	Chick liver	Chick kidney	Monkey kidney
Cystine	***	**	**	**
Histidine	**	*	*	**
Arginine	***	*	*	**
Tyrosine	*	*	*	*
Tryptophan	*	*	**	*
Valine	**	*	*	*
Isoleucine	**	**	**	*
Leucine	**	**	**	*
Serine	+	+	+	+
Glycine	+	+	+	+
Threonine	++	++	++	++
Alanine	+++	+	++	++
Methionine	+++	+	+	++
Lysine	**	+	+	+
Glutamine	+++	++	++	*
Aspartic acid	*	+	0	0
Hydroxyproline	*	0	0	0
Glutamic acid	*	+	0	++
Proline	+	+	**	*
Phenylalanine	**	**	**	0

[a] Changes based on the most active period of metabolism of each culture type.
+ = Slight increase, ++ = moderate increase, +++ = marked increase.
* = Slight decrease, ** = moderate decrease, *** = marked decrease.
0 = no detectable change.


E
D
B
A
1 B
2 B
M K
3 B
4 B
5 B
6 B
7 B

Comparative Pattern of Amino Acid Changes Caused by Different Tissue Cultures

Typical changes in the amino acid composition of the synthetic medium during growth of the different types of tissue cultures are summarized in Table IV. For comparative purposes, the changes previously established for chick embryonic heart cultures have been included in this table. It is evident that all four types of cultures utilize cystine, histidine, arginine, tyrosine, tryptophan, valine, isoleucine, and leucine. Similarly, all four tissues cultivated actively produce serine, glycine, threonine, alanine, and methionine. Differences among the cultures were noted in the utilization of lysine, glutamine, aspartic acid, hydroxyproline, glutamic acid, proline, and phenylalanine. From these results, it would appear that certain similarities exist in the amino acid metabolism of the various tissues cultivated, but that specific points of difference occur that may be related to the cell type under cultivation.

Discussion

Despite recent interest in the nutritional requirements of mammalian cells cultivated *in vitro*, relatively little information is available on the metabolic activity of such tissue cultures. Westfall, Peppers, and Earle (29) have followed amino acid changes in a complex medium during cultivation of strain HeLa cells and noted the accumulation of alpha-keto acids. Markert (12) has investigated the utilization of C^{14}-labelled glucose by a variety of chick embryonic tissues and found that, although the cellular constituents made from the labelled substrate are qualitatively similar in distinctly different tissues, the quantitative proportions of these substances released into the medium seem to be characteristic for individual cell types. In preliminary work in this series (22), we have studied amino acid changes in a completely synthetic medium during cultivation of chick embryonic heart tissue and have found that a characteristic pattern of amino acid uptake and accumulation occurs. This pattern of amino acid changes has subsequently been correlated with the amino acid requirements for survival of these cultures (16).

Characteristic patterns of amino acid uptake and accumulation have also been found with the various other types of cultures studied in the present investigation. In the case of chick embryonic kidney and liver, and also in the case of monkey kidney cultures, the amino acid changes in the medium

FIG. 3. Changes in adenine content of synthetic medium M 150 during cultivation of monkey kidney cells. Chromatogram run in *n*-butanol – acetic acid – water solvent system and developed with Wood's chloride reagent. MK = unused M 150 control. Numbers 1 to 7 = successive changes of medium exposed to the cultures for 3 days before each change.

E = Adenine
D = Hypoxanthine
C = Chloride ions
B = Inorganic salts
A = Inorganic salts

were distinctly less marked than in the case of chick embryonic heart tissue. The low level of activity of chick embryonic liver and kidney was somewhat unexpected, since these tissues are ordinarily considered to be intensely active metabolically. It is possible that synthetic medium M 150, which was developed largely on the basis of experiments with chick embryonic heart cultures, may permit a prolonged "resting-state" type of survival but not support active metabolism.

Arginine has been shown (16) to be an essential amino acid for all tissue cultures so far tested. Accordingly, it is of interest that all cultures in the present investigation showed an active uptake of this amino acid from the growth medium. However, analysis of the paper chromatograms failed to show the presence of citrulline or ornithine in the culture fluids from chick tissues. Therefore it would appear that removal of arginine from the culture medium represents an active incorporation of this amino acid by these tissue cells. Degradation of arginine through the action of arginase does not appear to be involved in utilization of this amino acid, unless it is assumed that all traces of citrulline and ornithine are taken up by the cells. A slightly different behavior was noted in the case of the monkey kidney cultures, where disappearance of arginine from the medium was accompanied by the appearance of ornithine but not citrulline. Whether this difference between monkey and chick kidney cultures represents a major species difference in metabolism remains to be determined.

The sulphur amino acid metabolism is of considerable interest in that all tissues studied showed a marked uptake of cystine from the culture medium accompanied by an increase in the methionine region. This observation is in accord with previous findings with chick embryonic heart tissue (14,15) which have established an essential requirement for cystine but only a supplementary requirement for methionine. It should be emphasized that the increase in the methionine region cannot be attributed definitely to methionine at the present time, since other uncharacterized substances may have been elaborated by the cells. However, the location of this spot in the exact methionine region of chromatograms developed both by acidic and neutral solvent systems suggests strongly that it is methionine.

Characteristic differences between the amino acid metabolism of various tissue cultures have been established in the present study, in addition to certain general similarities. Thus, chick embryonic heart tissue is characterized by a strong uptake of the basic amino acids and by slight release of glycine, aspartic acid, and glutamic acid. Chick embryonic kidney shows a pattern resembling that of chick embryonic liver except that a moderate uptake of proline occurs and no release of aspartic and glutamic acids can be detected. Monkey kidney cells proved to be distinctly different from the three chick embryonic tissues studied in that glutamine was utilized to a minor degree and no uptake of phenylalanine was found.

Utilization of glutamine by monkey kidney cells under these experimental conditions is of considerable interest since, in general, cultures of normal

tissues fail to utilize this amide while cultures of malignant cells appear to utilize it readily (to be published). These findings are in agreement with those of Roberts and Borges (25), who have demonstrated the release of glutamine into the surrounding fluids by regressing tumors but not by actively-developing tumors. The observation that trypsinized monkey kidney cells utilize glutamine suggests that the procedure of trypsinizing the tissue to form a cell suspension for rapid cultivation may have brought about a transformation from a normal to a malignant cell type, a change that has been observed to occur in many types of tissue cultures (8).

While each type of culture studied exhibited a different pattern of amino acid changes in the medium, a common pattern of purine metabolism was indicated. All four tissues employed in these experiments showed a high rate of uptake of adenine and the release of small amounts of material in the hypoxanthine region. While the concentrations of guanine and xanthine in the culture medium were too low to be detected by the method employed, it has been shown previously that guanine is poorly utilized by tissue cultures and that adenine and hypoxanthine are the two purines actively incorporated (10,11). On the basis of the present experiments, therefore, it would appear unlikely that qualitative differences exist in the purine metabolism of different types of tissue cultures.

In all studies on tissue culture nutrition and metabolism, the relationship of the cells under cultivation to the tissue of origin is open to some question. Thus, comparative enzymatic studies on a cultured cell line derived from liver and on freshly-prepared hepatic parenchymal cells (23) have shown entirely different metabolic behavior. Similarly, nutritional variants have been demonstrated to occur in human carcinoma cells (24) and in rabbit fibroblasts (6). In most cases, these variations in nutritional and metabolic properties have been demonstrated in cell lines under continuous and rapid propagation. For this reason, freshly-explanted tissues were employed in the present study and were maintained under conditions supporting survival rather than rapid propagation. It is hoped that information derived in this manner may establish the metabolic patterns of normal tissue cultures and may make it possible to detect differences between normal and malignant cells in tissue culture.

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NOTES

A NOTE ON THE EFFECT OF COLD AND VITAMIN C ON THE BLOOD LEVELS OF PHENYLALANINE AND TYROSINE IN RATS[1]

P. V. RANGNEKER[2] AND L. P. DUGAL

It has been shown that ascorbate has some beneficial effects (2, 3, 4) in animals exposed to cold. Besides, a number of biochemical interrelationships between ascorbic acid and amino acids (5, 8, 9, 11) have been reported *in vitro*. The present communication gives a comparison of the free amino acids in blood and urine between animals exposed to cold and those at room temperature. Observations are also made on the effect of ascorbate administration on the concentration of amino acids.

Method

Protein-free alcohol extracts were prepared and chromatographed on paper (1, 12). The conventional two-dimensional and Rutter's (10) technique of circular chromatography as modified by Giri and Rao (6) were used. The

TABLE I

PLASMA AMINO ACIDS IN MALE RATS (FIVE ANIMALS IN EACH GROUP) 72 HR. IN COLD ROOM (2.0±1.0° C.)

(Treatment: 75 mg. of ascorbate administered intraperitoneally twice a day for 2 weeks before they were sacrificed. Equal volumes of normal saline twice a day for controls for the same length of time. + sign indicates medium ninhydrin color (12, with slight modification). ++ sign indicates doubled intensity of ninhydrin color. − sign indicates absence.)

	Cold		Room temperature	
Amino acid	Ascorbate treated	Saline treated	Ascorbate treated	Saline treated
Phenylalanine	−	−	Traces	+
Tyrosine	−	Traces	+	++

TABLE II

AMINO ACIDS IN 24-HOUR URINE SAMPLES OF MALE RATS (FIVE ANIMALS IN EACH GROUP) 72 HR. IN COLD ROOM (0±1.0° C.)

(Treatment: 75 mg. of ascorbate administered intraperitoneally twice a day for 2½ weeks before they were sacrificed. Controls received equal volumes of normal saline for the same length of time. Sign interpretations as described for Table I)

	Cold		Room temperature	
Amino acid	Ascorbate treated	Saline treated	Ascorbate treated	Saline treated
Phenylalanine	−	−	−	+
Tyrosine	−	−	Traces	++

[1]This work was supported by a grant from the National Research Council of Canada.
[2]National Research Council Postdoctorate Fellow.

various amino acids were identified by running mixed chromatograms (6) and by using the known color tests. Twenty-four-hour samples of urine were chromatographed after they were desalted. Only those amino acids which showed marked changes under experimental conditions are represented in Tables I and II.

Results and Discussion

From Table I it is obvious that cold exposure results in complete disappearance of phenylalanine in ascorbate-treated rats as well as in controls. Furthermore it is evident that tyrosine disappears completely from the plasma of treated rats in the cold, but is present in traces in controls in the same environment. At room temperature there is a decrease in the plasma concentration of phenylalanine and tyrosine in those treated with ascorbate.

The 24-hour urine samples of rats in the cold room showed complete absence of tyrosine (Table II) and phenylalanine with or without ascorbate. The two amino acids are present in the urine samples of controls at room temperature. Phenylalanine is absent whereas tyrosine is present in traces in the urine of rats treated with ascorbate at room temperature.

We have also observed that in male rats exposed to cold and injected intraperitoneally with 50 mg. of L-tyrosine for a period of 17 days, the plasma and urine showed complete absence of tyrosine. Under the same conditions, the same amino acid was found to be present in the plasma and urine of controls at room temperature. Greene and Johnston (7) have made similar observations on the disappearance rate of tyrosine from the blood of dogs.

In a separate experiment we exposed guinea pigs to cold ($0° \pm 1.0°$ C.) for 72 hr. without any ascorbic acid supplement; and once again in these animals there was no trace of phenylalanine and tyrosine in the plasma.

From these observations it appears that in rats and guinea pigs the metabolism of phenylalanine and tyrosine is facilitated in a cold environment. Our findings suggest that ascorbate aids in the utilization of both phenylalanine and tyrosine in the rat kept at room temperature.

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Contents

Page

Properties of calf thymus histone isolated by three different methods—*L. B. Smillie, G. C. Butler, and David B. Smith* - - - - - - - 1

Studies on the enzymatic hydrolysis of sarin and tabun—*Peter A. Adie* - - 15

The intracellular localization of liver and kidney sarinase—*Peter A. Adie and Jules Tuba* - - - - - - - - - - - - - - - 21

Studies on the amino acids in the blood plasma of scorbutic guinea pigs—*P. V. Rangneker and L. P. Dugal* - - - - - - - - - - - 25

Accurate measurement of liquid intake of small animals—*R. J. G. Gillespie and C. C. Lucas* - - - - - - - - - - - - - - 29

An unexpected factor affecting the alcohol intake of rats—*R. J. G. Gillespie and C. C. Lucas* - - - - - - - - - - - - - - 37

Differences in nitrogen content in extracts of chilled and non-chilled tissues—*S. Fedoroff and R. Altschul* - - - - - - - - - - - - 45

The effect of stress and adrenalectomy on the response to dicumarol in rats—*G. J. Mogenson, L. M. Fisher, and L. B. Jaques* - - - - - - - 51

Uptake of radioactive sulphur by various tissues of normal and cholesterol-fed rabbits—*R. C. Buck and F. C. Heagy* - - - - - - - - - - 63

The estimation of maleic hydrazide by paper chromatography—*W. A. Andreae* 71

The properties of thrombinogen—*Edward Ronwin* - - - - - - - - 75

Oxidative metabolism of carbohydrates in insects. III. Hexose monophosphate oxidative cycle in the housefly, *Musca domestica* L.—*W. Chefurka* 83

The acute oral toxicity of spiramycin—*Eldon M. Boyd* - - - - - - 103

Limitations of the *in vitro* pituitary incubation system as an assay for ACTH-releasing activity—*Claude Fortier and Darrell N. Ward* - - - - - - 111

The role of the salivary duct system in the formation of the saliva—*A. S. V. Burgen and P. Seeman* - - - - - - - - - - - - - - 119

The hydrolysis of *p*-acetoxyphenylethylamines by insect cholinesterase—*L. S. Wolfe and G. D. Thorn* - - - - - - - - - - - - - 145

Preparation and molecular weight of γ-livetin from egg yolk—*W. G. Martin and W. H. Cook* - - - - - - - - - - - - - - - 153

The physiological activity of laminarin sulphate—*W. W. Hawkins and Verna G. Leonard* - - - - - - - - - - - - - - - - 161

The metabolism of animal tissues cultivated *in vitro*. II. Amino acid metabolism of chick embryonic kidney, chick embryonic liver, and monkey kidney cortex cultures—*Arthur E. Pasieka, Helen J. Morton, and Joseph F. Morgan* - - - - - - - - - - - - - - - - 171

Notes:

A note on the effect of cold and vitamin C on the blood levels of phenylalanine and tyrosine in rats—*P. V. Rangneker and L. P. Dugal* - - - 185